

Progesterone as a neuroprotectant in stroke

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ABSTRACT

Progesterone has been shown to be neuroprotective in a number of central nervous system injury models, including cerebral ischaemia. There is still a lack of understanding behind progesterone's neuroprotective properties, and the purpose of this project is to clarify some of these issues.

Osmotic mini-pump infusion was hypothesised to be more effective in delivering progesterone to the target organ of the brain, when compared to a bolus intraperitoneal injection. Progesterone pharmacokinetic profiles were compared between different dosing regimes. Intraperitoneal progesterone injection had a short half-life in both plasma and brain, while osmotic mini-pumps delivered higher concentrations of progesterone in plasma and particularly in brain, over a longer period, which supports the hypothesis.

It was hypothesised that progesterone will reduce NO production and cell death in *in vitro*. Progesterone reduced nitric oxide production after challenging microglia with LPS, which supports our hypothesis and the nuclear progesterone receptor was found not to have a major role in nitric oxide attenuation. Neither of the microglial cell lines, BV-2 and HAPI cells produced elevations in NO formation in ischaemic conditions. The *in vitro* oxygen and glucose deprived model of ischaemia, reduced viability in both microglial and neuronal cells. Also, high pharmacological concentrations of progesterone exacerbated ischaemic injury, which does not support the hypothesis of progesterone in reducing cell death.

Progesterone administration, via osmotic mini-pump infusion, was hypothesised to have a better outcome compared to vehicle treatment. After the onset of experimental stroke, progesterone delivery via osmotic mini-pump with loading dose was found to be beneficial in terms of

neurological deficit score in adult male mice, which supports the hypothesis. Also, we hypothesise that co-morbidity can affect the efficacy of progesterone treatment in outcomes. Aged animals have an increased sensitivity to experimentally induce stroke and did not display, in the outcomes measured, any benefit from progesterone treatment. NOD/ShiLtJ mice had severe symptoms, resulting in high mortality after surgery and are not recommended as a model of diabetes for experimental stroke. Hypertensive BPH/2 mice are a potential hypertensive model and had better functional outcomes after treatment with intraperitoneally administered progesterone, compared to non-treated hypertensive animals in our small preliminary study. This supports our hypothesis that co-morbidity can affect the efficacy of progesterone treatment in outcomes.

The gold-standard for assessing intervention effects across studies within and between subgroups is to use meta-analysis based on individual animal data. We hypothesise meta-analysis would reveal progesterone to reduce lesion volume, but also discover other effects in different subgroups of animals. Progesterone significantly reduced lesion volume, it also appeared to increase the incidence of death following experimental stroke. Furthermore, this negative effect appears to be particularly apparent in young ovariectomised female animals. These findings support the hypothesis that progesterone reduces lesion volume and progesterone having other effects in different subgroups.

This investigation has clarified some issues and expanded our understanding on the neuroprotective properties of progesterone. However, these findings indicate further investigation is still required before progesterone can be considered for use in clinical trials as a neuroprotectant in stroke.

PUBLICATIONS

Wong, R., Ray, D., and Kendall, D.A. (2012). Progesterone pharmacokinetics in the mouse: implications for potential stroke therapy. *J Pharm Pharmacol* 64, 1614-1620. (Published)

Sustained release of progesterone, enhances functional recovery following transient cerebral ischaemia in male mice (Submitted to journal)

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ABBREVIATIONS

5 α -DHP - 5 α -pregnane-3, 20-dione

AA - arachidonic acid

AChE - Acetylcholinesterase

ADP - Adenosine diphosphate

AE - Arterial embolism

AF - Atrial fibrillation

AIC - Akaike information criterion

Akt - Protein kinase B

AMPA – 2-amino-3-(hydroxyl-5-methyl-isoxazol-4-yl)propanoic acid

Ang - Angiotensin

ANOVA - Analysis of variance

ATP - Adenosine triphosphate

AUC - Area under the curve

BBB - Blood-brain barrier

BDNF - Brain derived neurotrophic factor

CAS - Carotid artery stenting

CAVATAS - *Carotid and vertebral artery transluminal angioplasty study*

CEA - Carotid endarterectomy

CHARISMA - Clopidogrel for high atherothrombotic risk and ischaemic stabilization, management, and avoidance

CI - Confidence interval

C max - Maximum recorded concentration

CNS - Central nervous system

COX-2 - Cyclo-oxygenase 2

CRP - C-reactive protein

CT - Computerised tomography

CYP (3A4,2D,2D6) - Cytochrome P450 (3A4, 2D, 2D6)

DECIMAL - Sequential-design, multicenter, randomized, controlled trial of early decompressive craniectomy in malignant middle cerebral artery infarction

DESTINY - Decompressive surgery for the treatment of malignant infarction of the middle cerebral artery

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

ECASS 3 - European cooperative acute stroke study

ECG - Electrocardiogram

ECST - European Carotid Surgery Trial

EDTA - Ethylenediaminetetraacetic acid

EIA - Enzyme immunoassay

ELISA - Enzyme-linked immunosorbant

eNOS - Endothelial nitric oxide synthetase

EVA-3S - Endarterectomy versus angioplasty in patients with symptomatic severe carotid Stenosis

ER-DP - Extended-release dipyridamol

ESPRIT - European/Australasian stroke prevention in reversible ischaemia trial

ESPS-2 - European stroke preventative study

ERK - Extracellular signal-regulated kinase

FBS - Foetal bovine serum

GABA_A - Gamma-aminobutyric acid A

GDNF - Glial cell line-derived neurotrophic factor

HAMLET - Hemicraniectomy after middle cerebral artery infarction with
life-threatening Edema trial

HAPI - Highly aggressively proliferating immortalised

HBSS - Hank's balanced salt solution

HCL - Hydrochloric acid

HDL - High density lipoprotein

HMG-CoA - 3-hydroxy-3-methyl-glutaryl-CoA

HRT - Hormone replacement therapy

IAD - Individual animal data

ICAM - Intercellular adhesion molecule

ICSS - *International carotid stenting study*

IFN γ - Interferon-gamma

IGF-1 - Insulin-like growth factor 1

IgG - Immunoglobulin G

IL - Interleukin

iNOS - Inducible nitric oxide synthase

I.P. - Intraperitoneal

LDL - Low density lipoprotein

LPS - Lipopolysaccharide

MATCH - Management of atherothrombosis with Clopidogrel in High- risk
patients

MAPK - Mitogen-activated protein kinase

MCA - Middle cerebral artery

MCAO - Middle cerebral artery occlusion

MI - Myocardial infarction

MMPs - Matrix metalloproteinases

MRI - Magnetic resonance imaging

MTT - ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

NADPH - Nicotine adenine dinucleotide phosphate

NASCET - North American Symptomatic Carotid Endarterectomy Trial

NK - Natural killer

NMDA - *N*-Methyl-D-aspartic acid

nNOS - Neuronal nitric oxide synthetase

NO - Nitric oxide

NOD - Non-obese diabetic

NOS - Nitric oxide synthetase

NX-059 - Disufenton sodium

OGD - Oxygen and glucose deprivation

PAF - Platelet activating factor

PBS - Phosphate-buffered saline

PGRMC - Progesterone receptor membrane component

PLA₂ - phospholipase A₂

PR (A, B) - Progesterone receptor (A, B)

PRE - Progestin response elements

PRKO - Progesterone receptor knock-out

PROactive - Prospective pioglitazone clinical trial in microvascular events

Progesterone-HRP conjugate - Horseradish peroxidase enzyme linked progesterone

PROGINS - Progesterone receptor gene polymorphism

PROGRESS - Perinopril protection against recurrent stroke study

RANTES - Regulated on activation, normal T cell expressed cell expressed and secreted

Resazurin - 7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide

ROS - Reactive oxygen species

SAH - Subarachnoid haemorrhage

SAINT - Stroke-acute ischaemic NXY treatment

SD - Standard deviation

SE - Standard error

SEM - Standard error mean

SFM - Serum-free media

SMC - Smooth muscle cell

SPACE - Stent-protected angioplasty versus carotid endarterectomy

SPARCL - Stroke prevention by aggressive reduction in cholesterol Levels

STAIR - Stroke therapy academic industry roundtable

TBI - Traumatic brain injury

TGF - Transforming growth factor

TIA - Transient Ischaemic Attack

T max - Maximum concentration time

TNF - Tumour necrosis factor

TOAST - Trial of ORG 10172 in Acute Stroke

t-PA - Tissue plasminogen activator

TxA₂ - Thromboxine A₂

VSCC - voltage-sensitive Ca²⁺ channels

WHI - Women's Health Initiative

%B - % Sample or standard bound

B₀ - Maximum bound

LIST OF CONTENTS

ABSTRACT	i
PUBLICATIONS	iii
ACKNOWLEDGMENT	iv
ABBREVIATIONS	v
LIST OF CONTENTS	xi
LIST OF FIGURES	xx
LIST OF TABLES	xxiv
1. CHAPTER 1: Introduction	1
1.1 INTRODUCTION	2
1.1.1 Definition of stroke.....	2
1.1.2 Types of stroke.....	2
1.1.2.1 Ischaemic stroke.....	2
1.1.3 Haemorrhagic stroke.....	3
1.1.4 Epidemiology.....	4
1.1.4.1 Statistics.....	4
1.1.4.2 Prevalence.....	4
1.1.5 Risk factors.....	5
1.1.6 Atherosclerosis.....	7
1.1.7 Artherothrombosis.....	9

1.1.8 Pathophysiology of ischaemic stroke.....	10
1.1.9 Symptoms and diagnosis.....	15
1.1.9.1 Focal neurological and ocular symptoms.....	15
1.1.10 Diagnosis.....	17
1.1.10.1 Brain imaging with CT and MRI.....	17
1.1.10.2 Carotid ultrasound (Doppler ultrasound).....	17
1.1.10.3 Electrocardiogram.....	18
1.1.10.4 Echocardiography.....	19
1.1.11 Acute ischaemic stroke treatments.....	19
1.1.11.1 Thrombolysis.....	19
1.1.11.2 Aspirin.....	20
1.1.11.3 Stroke units.....	20
1.1.11.4 Hemicraniectomy.....	21
1.1.12 Preventative ischaemic stroke treatments.....	22
1.1.12.1 Antiplatelet therapy.....	22
1.1.12.2 Cholesterol lowering.....	23
1.1.12.3 Carotid endarterectomy and stenting.....	23
1.1.13 Sex differences in physiology outcome and treatment for stroke.....	24
1.1.14 Female sex steroids for neuroprotection.....	25
1.2.1 Progesterone.....	28

1.2.2 Progesterone as a candidate for neuroprotection.....	29
1.2.3 Translational perspective on progestogens for ischaemic stroke....	33
1.2.4 Progesterone in the brain.....	34
1.2.5 Polymorphisms in progesterone receptors and enzymes.....	36
1.2.6 Allopregnanolone.....	36
1.2.7 Considerations for progesterone’s mechanism for neuroprotection.....	38
1.3 Research with animal models.....	39
1.3.1 Choice of ischaemic model.....	40
1.3.2 Choosing the species.....	43
1.3.3 Physiologically relevant models.....	43
1.3.4 Limitations of using experimental stroke models.....	44
1.4 Clinical trials for neuroprotection.....	45
1.5 AIMS	47
2. CHAPTER 2: Pharmacokinetics in the mouse: implications for potential stroke therapy.....	50
2.1 INTRODUCTION.....	51
2.2 MATERIALS AND METHODS.....	53
2.2.1. Ether extraction protocol.....	53
2.2.2 Animals.....	54
2.2.3 Progesterone administration.....	54

2.2.4 Determination of progesterone in plasma and brain.....	55
2.2.5 Data analysis.....	56
2.3 RESULTS	57
2.4 DISCUSSION	65
3. CHAPTER: Effects of progesterone and oxygen glucose deprivation on microglial and neuroblastoma cells	72
3.1 INTRODUCTION	73
3.2 MATERIALS AND METHODS	76
3.2.1 Maintaining SHSY5Y and BV-2/HAPI cell culture stocks.....	76
3.2.2 LPS experiments.....	77
3.2.3 OGD experiments.....	77
3.2.3.1 Cell Preparation.....	77
3.2.3.2 OGD and controls.....	78
3.2.3.3 Re-oxygenation.....	78
3.2.3.4 Assessment of cell viability.....	79
3.2.3.5 Resazurin viability assay.....	79
3.2.3.6 MTT viability assay.....	80
3.2.3.7 Trypan blue with automated cell-counter viability assay.....	81
3.2.3.8 Neutral red viability assay.....	81
3.2.3.9 Nitric Oxide measurements.....	82
3.3 RESULTS	83

3.3.1 Nitric oxide production and viability of BV-2 microglia in OGD conditions.....	83
3.3.2 Nitric oxide production and viability of HAPI microglia in OGD conditions.....	87
3.3.3 Nitric oxide production and viability of BV-2 microglia in the presence of LPS.....	88
3.3.4 Nitric oxide production and viability of BV-2 microglia in the presence of LPS, progesterone and mifepristone.....	91
3.3.5 SHSY5Y neuronal cell line viability analysis with trypan blue (cell counter) viability assay.....	94
3.3.6 The effect of mifepristone on SHSY5Y cells in OGD conditions using the trypan blue viability assay.....	100
3.4 DISCUSSION	105
4. CHAPTER 4: Progesterone treatment and focal ischaemia on mouse models	118
4.1 INTRODUCTION	119
4.2 MATERIALS AND METHODS	127
4.2.1 Animals.....	127
4.2.1.1 Adult C57 Bl/6.....	128
4.2.1.2 Aged C57 Bl/6.....	128
4.2.1.3 NOD/ShiLtJ.....	128
4.2.1.4 BPH/2 hypertensive mice.....	129
4.2.2 Focal cerebral ischaemia.....	130

4.2.3 Drug treatment.....	132
4.2.4 Assessment of general well-being.....	132
4.2.5 Neurological deficit score.....	133
4.2.6 Foot fault.....	135
4.2.7 T-maze.....	135
4.2.8 Brain oedema.....	136
4.2.9 Statistical Analysis.....	137
4.3 RESULTS	137
4.3.1 Adult C57 Bl/6.....	137
4.3.1.1 Assessment of general well being in adult C57 Bl/6.....	138
4.3.1.2 Neurological score for adult C57 Bl/6.....	141
4.3.1.3 Foot-Fault for adult C57 Bl/6.....	142
4.3.1.4 T-maze for adult C57 Bl/6.....	143
4.3.1.5 Modified analyses of outcomes in adult male mice to consider death.....	144
4.3.2 Effect of aging on outcome following MCAO and progesterone treatment.....	148
4.3.2.1 Neurological score for aged animals.....	151
4.3.2.2 Oedema at time of death for aged C57 Bl/6.....	152
4.3.3 Non-obese diabetic mice (NOD/ShiLtJ).....	153
4.3.4 Hypertensive BPH/2 mice.....	156

4.3.4.1 Hypertensive BPH/2 with increasing occlusion times.....	156
4.3.4.2 Progesterone in hypertensive BPH/ 2 animals and MCAO.....	162
4.3.4.3 Modified analyses of outcomes in hypertensive BPH/2 mice to consider death.....	167
4.4 DISCUSSION	169
5. CHAPTER 5: Progesterone treatment for experimental stroke: an individual animal meta-analysis.....	181
5.1 INTRODUCTION	182
5.2 METHODS	182
5.2.1 Ethics.....	182
5.2.2 Search strategy.....	182
5.2.3 Selection of studies.....	183
5.2.4 Validity assessment.....	183
5.2.5 Data abstraction.....	184
5.2.6 Data extraction.....	184
5.2.7 Quantitative data synthesis.....	185
5.3 RESULTS	187
5.3.1 Study flow.....	187
5.3.2 Study characteristics.....	189
5.3.3 Quantitative data synthesis.....	195
5.3.3.1 Lesion volume – combined individual and summary data.....	195

5.3.3.2 Adjustments for analyses involving individual animal data.....	198
5.3.3.3 Lesion volume – individual animal data, death included.....	198
5.3.3.4 Lesion volume – individual animal data, death excluded.....	199
5.3.3.5 Death.....	200
5.3.3.6 Progesterone blood concentration.....	203
5.4 DISCUSSION	204
6. CHAPTER 6: General Discussion	211
6.1 GENERAL DISCUSSION	212
7. APPENDIX	226
7.1 Pharmacokinetics Method development.....	226
7.1.1 Progesterone ELISA kit from Alphadiagnostic.....	226
7.1.2 Serum albumin/IgG removal.....	229
7.1.3 Progesterone enzyme immunoassay kit from Cayman Chemicals.....	231
7.1.4 Ether extraction development.....	233
7.2. Method development for SHSY5Y viability.....	236
7.2.1. MTT viability assay.....	236
7.2.2. Resazurin viability assay.....	238
7.2.3. Viability of SHSY5Y using the resazurin assay.....	239
7.2.4. Viability comparison of resazurin assay with trypan blue (cell counter) in SHSY5Y.....	242

7.3.1 Invitation letter template to authors for individual animal meta-analysis	
collaboration.....	244
7.3.2 Document outlining individual animal data meta-analysis to authors.....	247
8. REFERENCES	254

LIST OF FIGURES

CHAPTER 1

1.1: Cascade of biochemical events leading to apoptosis or necrosis following cerebral ischaemia.....	11
1.2: Female/Male stroke incidence rate ratios for different age bands with 95% CIs.....	26

Chapter 2

2.1: Elimination of progesterone from the brain after one bolus intraperitoneal dose of 8mg/kg.....	58
2.2: Brain progesterone concentrations in mini-pump-implanted male C57 Bl/6 mice injected with 8mg/kg of progesterone after implantation.	61
2.3: Plasma progesterone concentrations in mini-pump-implanted male C57 Bl/6 mice injected with 8mg/kg of progesterone after implantation.	62
2.4: Progesterone concentration in difference brain regions after mini-pump implantation.....	63
2.5: Distribution of progesterone in whole brain after mini-pump implantation.....	64

CHAPTER 3

3.1: BV-2 viability after 4 hrs in normoxic conditions.....	83
3.2: BV-2 viability after 4 hrs in OGD.....	84

3.3: BV-2 viability after 6hrs treatment in OGD followed by 24 hrs re-oxygenation.....	85
3.4: BV-2 viability after 8 hrs of OGD followed by 24hrs of re-oxygenation.....	86
3.5: HAPI viability after 6hrs treatment in OGD followed by 24 hrs re-oxygenation.....	88
3.6: Viability of BV-2 controls.....	89
3.7: Nitric oxide production of BV-2 controls.....	90
3.8: Viability of BV-2 cells in the presence of LPS and increasing concentrations of progesterone.....	92
3.9: Viability of BV-2 cells in the presence of LPS plus mifepristone and treated with increasing concentrations of progesterone.....	93
3.10: Nitric oxide production in BV-2 microglial cells exposed to LPS with and without mifepristone and treated with increasing concentrations of progesterone.....	94
3.11: SHSY5Y viability of vehicle and progesterone in normoxic conditions over time using the trypan blue assay.....	96
3.12: SHSY5Y viability in OGD conditions over time using the trypan blue assay.....	97
3.13: SHSY5Y viability after 6 hrs OGD, in the presence of different concentrations of progesterone using the trypan blue assay.....	98
3.14: SHSY5Y viability after 8 hrs OGD, in the presence of different concentrations of progesterone using the trypan blue assay.....	99

3.15: SHSY5Y viability of vehicle and progesterone, with increasing exposure to OGD using the trypan blue assay.....	100
3.16: SHSY5Y viability in the presence of mifepristone and increasing progesterone concentrations, after 6hrs exposure to OGD using the trypan blue assay.....	101
3.17: SHSY5Y viability with and without the presence of mifepristone and increasing progesterone concentrations, after 6hrs exposure to OGD using the trypan blue assay.....	102
3.18: No effect of mifepristone on attenuated SHSY5Y viability in the presence of progesterone (100µM), after 6hrs exposure to OGD using the trypan blue assay.....	103

CHAPTER 4

4.1: Comparison of post-surgery survival according to treatment/surgery of adult C57 Bl/6 mice.....	139
4.2: Weight loss of pre-surgery weight of adult C57 Bl/6.....	140
4.3: Neurological deficit score for adult C57 Bl/6.....	141
4.4: Foot fault for adult C57 Bl/6.....	142
4.5: Modified weight loss of pre-surgery weight for adult C57 Bl/6.....	146
4.6: Modified neurological deficit score for adult C57 Bl/6.....	147
4.7. Foot fault for adult C57 Bl/6 adjusted for deaths.....	148
4.8: Comparison of post-surgery survival according to treatment/surgery of aged C57 Bl/6 mice.....	150
4.9: Weight loss of aged C57/Bl6 24h post-MCAO.....	151

4.10: Neurological score for aged C57/Bl6 24h post-MCAO.....	152
4.11: Oedema at time of death for aged C57 Bl/6.....	153
4.12: NOD/ShiLtJ weight over time of monitoring.....	154
4.13: NOD/ShiLtJ water consumption over time of monitoring.....	155
4.14: Survival of BPH/ 2 hypertensive mice with increasing occlusion time.....	157
4.15: Weight Loss from pre-surgery weight of BPH/ 2 hypertensive animals with increasing occlusion time.....	158
4.16: Neurological deficit score for BPH/2 with increasing occlusion times.....	159
4.17: Foot fault for BPH/2 with increasing occlusion times.....	159
4.18: Oedema of animals receiving increased occlusion that survived to day 7.....	160
4.19: The percentage of BPM/2 animals that survived post surgery over time.....	163
4.20: Weight loss from pre-surgery weight of BPH/2.....	164
4.21: Neurological score for BPH/2.....	164
4.22: Foot fault for BPH/2.....	165
4.23: T-maze % alternation for BPH/2.....	165
4.24: T-maze % left/right ratio for BPH/2.....	166
4.25: T-maze for time taken to complete trials for BPH/2.....	166
4.26: Oedema of BPH/2 that survived to day 7.....	167

4.27: Modified neurological deficit Score for BPH/2.....	168
4.28: Modified foot fault for BPH/2.....	169

CHAPTER 5

5.1: Flow chart showing study search, and included and excluded studies.....	188
5.2: Begg's funnel plot for studies of progesterone on lesion volume when combining individual animal and summary data.....	194
5.3: Forrest plot of effect of progesterone on lesion volume using individual animal and summary data.....	197
5.4: Forrest plot of effect of progesterone on death using individual animal and summary data.....	202
5.5: Relationship between blood concentration and loading dose of progesterone.....	203

APPENDIX

7.2.1: Comparison of MTT and resazurin assay controls after 2 hrs of OGD.....	237
7.2.2: SHSY5Y viability after 3 hrs of OGD using MTT assay.....	238
7.2.3: SHSY5Y viability after 2 hrs of OGD, followed by incubation with resazurin at 1 and 2 hrs before assay.....	239

7.2.4: SHSY5Y viability of OGD controls with increasing incubation time using resazurin assay.....	240
7.2.5: Viability of SHSY5Y cells exposed to increasing concentrations of progesterone during 4 hrs of OGD, with or without 24 hrs of re- oxygenation using resazurin assay.....	241
7.2.6: SHSY5Y viability of OGD controls over time: comparison of resazurin and trypan blue assays.....	243

LIST OF TABLES

CHAPTER 1

1.1: Advantages and disadvantages of different focal ischaemic models.	42
--	----

CHAPTER 2

2.1: Progesterone concentrations in plasma and brain, following a single bolus i.p. dose of 8mg/kg.....	59
---	----

CHAPTER 3

3.1: Overview of main experimental results.....	104
---	-----

CHAPTER 4

4.1: Outcomes found in co-morbid models of stroke in comparison to healthy adult animals.....	126
4.2: Type and overall number of mice in each experimental group that underwent surgery.....	128
4.3: Neurological score (28 points).....	134
4.4: Number of mice in each experimental group used for weight loss that underwent surgery for adult C57 Bl/6.....	139
4.5: Number of mice in each experimental group used for behavioural testing for young C57 Bl/6.....	140
4.6: T-maze summary for adult C57 Bl/6.....	143
4.7: Number of mice in each experimental group used for modified weight loss analysis for adult C57 Bl/6.....	145

4.8: Number of BPH/ 2 mice in each experimental group used for increasing occlusion times.....	157
4.9: T-maze for BPH/2 with increasing occlusion times.....	160
4.10: Number of BPH/ 2 mice in progesterone treated and non-treated experimental group.....	162

CHAPTER 5

5.1: Studies included in the analysis.....	190
5.2: Experimental Results.....	192
5.3: Excluded studies and reasons for exclusion after meeting inclusion criteria.....	194
5.4: Effect of progesterone on standardised total lesion volume using combined individual animal and summary data, both combined and in pre-defined subgroups.....	196
5.5: Effect of progesterone on standardised total lesion volume, including animals that died, using individual animal data overall and in pre-defined subgroups.....	199
5.6: Effect of progesterone on standardised total lesion volume, excluding animals that died, using individual animal data overall and in pre-defined subgroups.....	200
5.7: Effect of progesterone on death using individual animal *and summary* data overall and in pre-defined subgroups.....	201

Appendix

7.1.1: Plasma controls with Alphadiagnostic assay.....	228
7.1.2: Plasma controls through ether extraction with Alphadiagnostic assay.....	229
7.1.3: Plasma with HCL and ether extraction.....	229
7.1.4: Plasma albumin/IgG removal and ether extraction.....	231
7.1.5: Progesterone-spiked plasma and brain readings with progesterone EIA kit.....	233
7.1.6: Ether extraction 1x of progesterone from brain homogenate (%)	234
7.1.7: Ether extraction 3x of progesterone from brain Homogenate (%).....	235
7.1.8: Ether extraction 3x of progesterone standards (%).....	235

CHAPTER 1

Introduction

1.1 INTRODUCTION

1.1.1 Definition of stroke

Stroke is defined as an acute loss of focal brain function caused by either rupture of a blood vessel of the brain (haemorrhagic stroke), or caused by inadequate blood supply to a part of the brain due to a blockage of a blood vessel (ischaemic stroke) (Hankey, 2007).

1.1.2 Types of stroke

1.1.2.1 Ischaemic stroke

Approximately 80% of strokes are ischaemic and are caused by a sudden interruption of blood flow within a cerebral artery due to a blood clot (thrombosis) or a detached intravascular mass (embolism) (Donnan et al., 2008). The brain requires a constant supply of oxygen and glucose via the blood and is highly sensitive to blood flow changes. Any interruption of the blood supply to an area of the brain for more than a few minutes will stop the affected area from functioning properly (Mehta et al., 2007). If oxygen and glucose supply is not restored, permanent damage may result within 5-6 minutes (Hutchins and Barger, 1998).

The Ischaemic stroke subtype is a significant predictor for long term survival according to the 'Trial of ORG 10172 in Acute Stroke Treatment' (TOAST) criteria (Madden et al., 1995). In a small study, it was found that the incidence rate of large artery atherosclerosis is more than twice as high in men as compared to women in the European population (age adjusted) and small artery occlusion patients are three times more likely to be alive as compared to patients with cardio embolism after two years of onset

(Kolominsky-Rabas et al., 2001). Ischaemic stroke subtypes includes: cardio embolism, small artery occlusion, large artery atherosclerosis, stroke of other determined cause and stroke of undetermined cause (due to two or more causes identified, negative evaluation and incomplete evaluation) (Donnan et al., 2008).

Transient Ischaemic attack (TIA) of the brain can be described as an acute loss of focal brain or monocular function, which lasts less than 24 hours. The clinical syndrome is thought to occur due to inadequate blood supply to a part of the brain or eye due to low blood flow, thrombosis or embolism associated with diseases of blood vessels, the heart or blood. In this respect it is similar to an ischaemic stroke, however symptoms are resolved within a 24 hour period (Hankey, 2007). TIA is also a risk factor, which increases the chances for eventually developing a stroke (Ferro et al., 1996).

1.1.3 Haemorrhagic stroke

The majority of strokes are caused by ischaemia as mentioned previously, while the remainder are caused by haemorrhage. Haemorrhagic stroke is characterised by a "burst" artery resulting in blood bleeding out into brain tissue (intracerebral haemorrhage) or over the brain surface area (subarachnoid haemorrhage). A rupture to the arterial wall will result in blood "spurting" out at high pressure and can tear brain tissue, forming a mass of blood (haematoma). Alternatively, a haematoma can slowly form as a result of blood seeping out over a prolonged period. Haematoma puts pressure on the normal surrounding brain and prevents the surrounding area from receiving blood from other surrounding blood vessels. As a result, the tissue surrounding the haematoma may die (Hankey, 2007).

1.1.4 Epidemiology

1.1.4.1 Statistics

In the UK the third leading cause of death after heart disease and cancers is stroke (Stroke Association, 2003), and causes a greater range and impact of disability compared to any other condition (Adamson J., 2004). In England, a minimum of 450,000 people are severely disabled from the condition (2005). Approximately every 5 minutes in the UK someone has a stroke, which adds up to an estimate of 150,000 people a year. A quarter of strokes each year in the UK happen to people under the age of 65 and approximately 10,000 happen to those under 30. In total, 1,000 occur in children and babies (2008). The risk of a recurrent stroke is between 30-43% by 5 years, following the first incidence (2008). Globally, stroke care consumes in the region of 2-4% of the total health care costs and in industrialised countries stroke care can account for more than 4% of direct health care costs (Donnan et al., 2008).

1.1.4.2 Prevalence

The prevalence of stroke in the population is dependent on age and gender. Male stroke prevalence is 41% higher than females, with very large variation between age bands and populations (Appelros et al., 2009). Stroke prevalence is also dependent on incidence and survival. The incidence of stroke can vary between the populations in countries. A study of the incidence of stroke in Europe found higher rates on average in eastern countries, while lower rates were observed in more southern European countries (Heuschmann et al., 2009). Different ethnic groups also have different incidences of strokes and its subtypes (Schneider et al.,

2004). Such differences are partly due to genetic susceptibility and variations in diet and life style.

Mortality rates vary depending on factors such as incidence, aetiological subtypes, case-fatality and severity of stroke, as well as age and sex. Stroke mortality rises substantially with age and can vary within countries and even within large cities. In general, stroke mortality is falling with the exception of Easton Europe and may be due to the decline in the incidence of stroke and improved survival rates. As mentioned previously, the incidence of stroke is in decline, which may be because of environmental factors and so can be modified by improved diet, reduced smoking and better control of blood pressure. Even if the incidence of stroke is declining, it is still going to be substantial in the foreseeable future. If the decline is not at least 2% per annum every year then the absolute number of strokes will increase in view of the ageing population in western societies (Hankey, 2007).

1.1.5 Risk factors

Risk factors for ischaemic stroke include: high blood pressure, increasing age, male gender, smoking, low social status, diabetes, atrial fibrillation, ischaemic heart disease, TIA, carotoid stenosis, peripheral vascular disease, hyperlipidemia, hormonal treatment (e.g. oral contraceptive pill), post menopausal state, pregnancy, alcohol use, poor diet, obesity and genetic risk factors. Some of these risk factors are linked, including: obesity and blood pressure, while others can be reduced by lifestyle changes, such as diet and exercise.

Age is the single most important risk factor for stroke and the incidence of stroke increases with age (Feigin et al., 2003; Herman et al., 1982;

Niessen et al., 1993). For each successive 10 years after the age of 55, the stroke rate more than doubles in both men and women (Brown et al., 1996; Wolf et al., 1992). Stroke incidence rates are 1.25 times greater in men, but because women tend to live longer than men, more women than men die of stroke each year (Sacco et al., 1997). Stroke severity at onset and patient age are the most important factors for predicting prognosis (Ingall, 2004). In an epidemiology study, aged patients of over 80 years old, was found to have a higher proportion of conscious impairment at admission, a longer acute ward stay, a higher incidence of total anterior circulation infarct and a lower frequency of lacunar infarct (Lee et al., 2007).

High-risk patients with vascular risk factors, including hypertension and dyslipidaemia, are prone to stroke. Effective control of these risk factors remains the best therapy for reducing stroke burden (Toyoda, 2009). Hypertension is an established stroke risk factor and studies, such as the 'Perinopril protection against recurrent stroke study' (PROGRESS) helped to clarify this. Anti-hypertensive pharmacotherapy reduced the risk of recurrent stroke or TIA, including 11% of patients with intracerebral haemorrhage (PROGRESS Collaborative Group, 2001). A meta-analysis of seven randomised control trials, including PROGRESS also showed reduced risk of stroke by anti-hypertensive pharmacotherapy (Rashid et al., 2003). The 'Stroke prevention by aggressive reduction in cholesterol levels' (SPARCLE) study was the first to show the impact of lipid lowering therapy by statins in reducing the risk of recurrent stroke (Amarenco et al., 2006).

Diabetes mellitus is one of the most rapidly growing diseases in the world, affecting over 230 million people worldwide and with an estimated global prevalence of 5.1%. It is a major cause of excess cardiovascular morbidity and mortality in the Western world (Reimann et al., 2009). Diabetes can

be categorised into type 1 and type 2. Type 1 diabetes mellitus involves the autoimmune destruction of insulin-producing pancreatic beta-cells via auto-aggressive T-cells and pancreatic infiltration by macrophages. It accounts for approximately 10% of all diabetes cases (Achenbach et al., 2005; Foulis et al., 1991). The other 90% is Type 2 and is the most common endocrine disorder worldwide. Pathogenesis of type 2 involves abnormalities in glucose and lipid metabolism, inadequate insulin secretion from pancreatic beta-cells and resistance to insulin activity (insulin resistance) (Goldstein, 2007). Significant loss of beta-cell mass (>50%) over time is casually related to hyperglycemia (Butler et al., 2003).

Abnormally increased levels of homocysteine (Hyperhomocysteine), is an emerging risk factor for ischaemic stroke. However, the role of homocysteine is controversial. A study investigated if there was a correlation of homocysteine with stroke severity and clinical outcome, but did not find a link between elevated plasma homocysteine and the severity or outcome of stroke. However, they did find an association with ischaemic and haemorrhagic stroke. At higher levels, there was also an association with small artery stroke (Perini et al., 2005). Additional findings from the HOPE 2 trial, found lowering of homocysteine with folic acid and vitamins B6 and B12 reduced the risk of overall stroke. It did not however reduce the severity of stroke (Saposnik et al., 2009).

1.1.6 Atherosclerosis

Atherosclerosis is associated with acute ischaemic stroke and can lead to early mortality (Roquer et al., 2007). The development of atherosclerosis involves the hardening and thickening of the arterial wall, due to build up of fatty materials, such as cholesterol. Common arterial sites of

atherosclerosis are areas of haemodynamic sheers stress, turbulent blood flow, stagnation of blood, and sites of endothelial trauma. The main areas affected are mainly large and medium sized arteries at places of arterial branching, tortousity and confluence.

Lesions of artherosclerosis are characterised by focal areas of lipid accumulation and intimal smooth muscle cell (SMC) proliferation within the arterial wall. The pathogenesis of artherosclerosis is not fully clear, but is thought to involve altered functional or structural integrity of the endothelial barrier, thereby allowing a net influx of lipids from circulating plasma (Alexander et al., 1991). Low density lipoprotein (LDL) is oxidised by free radicals, such as reactive oxygen species (ROS), and is deposited within the arterial wall where it is absorbed by macrophages, which then become foam cells. The inflammatory response is evoked with T-cells and the production of cytokines, with platelet activating factor (PAF) as the crucial mediator of this inflammatory response and is synthesised by many cells involved in the development of artherosclerosis, including platelets, monocytes, foam cell-macrophages and endothelial cells. Both PAF and PAF-like lipids are associated with elevated oxidised LDL (Demopoulos et al., 2003). SMC proliferate with fibrosis occurring forming fibrolipid plaques. These plaques, also known as atheromatous plaques, invade the media and gradually spread along and around the arterial wall, resulting in narrowing of the arterial lumen. Plaques can become 'active' or 'unstable' from time to time and can become complicated plaques by erosion or fissuring of thin parts of the fibrous caps, haemorrhaging into the plaque, or rupture of the plaque. Subsequently, platelet adhesion, activation and aggregation result in thrombus formation. Alternatively, plaques may become calcified or necrotic. Heavily calcified or fibrotic plaques make the whole artery rigid, elongated, and tortuous and at times ectatic. Dilation or

distension from ectasia and aneurysmal bulging, particularly of the basilar artery can compress nearby structures, such as the lower cranial nerves and brainstem (thromboembolism may also result), although arterial rupture is uncommon (Hankey, 2007). Unable to process accumulating oxidised LDL, foam cell-macrophages grow and eventually rupture depositing more oxidised cholesterol into the artery wall. This activates more macrophages and results in a continuous cycle.

1.1.7 Artherothrombosis

The acute rupture of atherosclerotic plaque is usually the trigger that leads to ischaemic disease. Mechanical and immunological forces partly contribute to the vulnerability of plaque rupture. The rupturing of atherosclerotic plaque exposes collagen either within the plaque or in the sub-endothelium, which allows platelets to adhere to the arterial wall or leading to the activation of the platelets. Once activated, platelets secrete products such as adenosine diphosphate (ADP), serotonin and thromboxane A₂ that, themselves, promote further platelet activation, vasoconstriction and neointimal proliferation and, therefore, contribute further disease progression. Activated platelets produce other inflammatory modulators such as platelet derived growth factor, platelet factor 4, CD40 ligand, thrombospondin, transforming growth factor and nitric oxide (NO). Also, CCL5; 'regulated on activation, normal T cell expressed cell expressed and secreted' (RANTES) promotes the directed migration of leukocytes into damaged or inflamed tissue. Platelets are a likely source of RANTES, as they are known to release large amounts of the chemokine from α -granules once activated (Yilmaz et al., 2006). RANTES is also produced by other cells, including T-lymphocytes,

endothelial, smooth muscle and glial cells (Terao et al., 2008). Final steps in the aggregation of platelets involve the activation of the platelet glycoprotein IIb/IIIa (GPIIb/IIIa) receptor, which binds to fibrogen and in turn to other platelets. This leads to the formation of a platelet rich mural thrombus, with or without peripheral embolisation, leading to the manifestation of stroke (Chaturvedi and Yadav, 2006). Embolic blockade of the blood vessel is the most common cause of ischaemic stroke.

1.1.8 Pathophysiology of ischaemic stroke

The pathophysiology of ischaemic stroke involves a complex process involving excitotoxic, inflammatory, and microvascular mechanisms leading to tissue necrosis and apoptosis. Some areas of brain tissue are salvageable if reperfusion is initiated early before the processes become irreversible. After occlusion of a cerebral artery that supplies an area of the brain, the centre of this poorly perfused area forms a central core of necrotic tissue and is considered to be irreversibly damaged. Under normal physiological conditions, the normal cerebral blood flow is maintained approximately 50-60 ml/100g/min, but blood flow in the core drops to <7 ml/100g/min (Baron, 1999). This area is surrounded by a moderately ischaemic zone called the penumbra. It is characterised as an area of dysfunction, due to metabolic and ionic disturbances, but the structural integrity is preserved and so is potentially salvageable. The cerebral blood flow in this region is 7-17 ml/100g/min (Baron, 1999). If the area is not reperfused early enough, then the lack of oxygen and ATP availability will result in cellular apoptosis, such that the core region is likely to expand into the penumbra. With declining blood flow, functional synaptic activity is affected first and as ischaemia progresses, metabolic activity is then

red borders represent important events in the cascade. Blue borders indicate reperfusion (Sutherland et al., 2012). AA= arachidonic acid, AMPA= α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid, BBB= blood brain barrier, iNOS= inducible nitric oxide synthase, NADPH= nicotinamide adenine dinucleotide phosphate-oxidase), NMDA= N-methyl-D-aspartate, nNOS= neuronal nitric oxide synthase, NO= nitric oxide, PLA₂= phospholipase A₂, VSCC= voltage-sensitive Ca²⁺ channels.

A decrease in blood flow after vessel occlusion, promotes a complex biochemical cascade in the ischaemic tissue, leading to activation of multiple pathways dependent on the intensity and duration of ischaemia. The events include glutamate mediated cytotoxicity, involving *N*-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(hydroxyl-5-methyl-isoxazol-4-yl)propanoic acid (AMPA)/kainate receptors, calcium overload, oxidative stress, stress signalling, neurovascular pathophysiology and inflammation, cell death and gene expression (Mehta et al., 2007). This biochemical cascade interferes with glucose metabolism and causes cell sodium pump failure, leading to neuronal depolarisation (Hutchins and Barger, 1998). As a result, the influx of calcium leads to the activation of calcium dependent enzymes such as calpain, endonucleases, ATPases, phospholipases and the generation of free radicals, such as ROS, eventually resulting to excitotoxic cell death (see figure 1.1 for detailed time course of events). Apoptosis in the penumbra can go through three stages: activation, commitment and execution. Activation involves rising calcium concentrations and ROS formation, promoting apoptotic gene expression. The commitment stage is the point of no return and is thought to involve mitochondria, regulated by changes in permeability of their outer membrane. In the final stage, degradation enzyme complexes including caspases, calpains and endonucleases play an active role. It is thought that preventing the first

two stages, might avoid neuronal damage. Acting on the last stage might only delay but not impede cell death. All of these different complex processes are thought to be potential targets for neuroprotection (Jordan et al., 2007).

Inflammatory events, which are initiated a few hours following focal ischaemia is characterised by peripheral leukocyte influx into the cerebral parenchyma and activation of endogenous microglia (Zheng and Yenari, 2004), underlie the transition from ischaemic to inflammatory injury. The interruption of cerebral blood flow leads to energy depletion and necrotic cell death, which can trigger immune responses that ultimately lead to inflammatory cell activation and infiltration. Reperfusion of the occluded vessel (by compensation of the collateral circulation or spontaneous or therapeutic recanalisation of the occluded vessel causes) leads to generation of ROS either by reperfusion of oxygenated blood or production within brain and immune cells. The generation of ROS then stimulates ischaemic cells, including ischaemic neurons to secrete inflammatory cytokines and chemokines, which upregulates adhesion molecules (selectins, integrins and immunoglobulins) in the cerebral vasculature and peripheral leukocyte recruitment, respectively. Activation of inflammatory cells can release a variety of cytotoxic agents, including more cytokines, matrix metalloproteinases (MMPs), NO and more ROS. These substances may induce more cell damage as well as disruption of the blood brain barrier (BBB) and extracellular matrix (Danton and Dietrich, 2003). BBB disruption can further potentiate injury and secondary ischaemic injury by allowing serum elements and blood to enter the brain (Rosenberg et al., 2001). This secondary damage development is a consequence of brain oedema, post-ischaemic microvascular stasis and vasomotor/haemodynamic deficits leading to hypoperfusion and post-

ischaemic inflammation, involving activation of microglia and brain infiltration of peripheral inflammatory cells. Migration of periphery circulating inflammatory leukocytes can amplify the inflammatory signal cascade, which enhances damage (Dirnagl et al., 1999).

Focal cerebral ischaemia and post-ischaemic reperfusion causes cerebral capillary dysfunction, results in progressive alteration to the permeability of the BBB, leading to oedema formation and haemorrhagic conversion. When the capillaries that form the BBB can no longer retain the intravascular constituents, such as Na^+ , water, serum proteins and blood, these substances enter the extracellular space of the brain, causing swelling. Oedema can be divided into different subtypes that can progress through cytotoxic oedema, ionic oedema, vasogenic oedema to eventual haemorrhagic conversion (Simard et al., 2007). Cytotoxic oedema is a pre-morbid process involving oncotic swelling of cells due to the movement of osmotically active molecules (principally Na^+ , Cl^- and water) from the extracellular to the intracellular space. Depending on the severity of ischaemia, cytotoxic oedema may take place within minutes, or hours after stroke. Oncotic death of neurons is the ultimate consequence of cytotoxic oedema (Kimelberg, 1995). Ionic oedema results from cytotoxic oedema of endothelial cells, due to expression of cation channels both the luminal and abluminal side, allowing Na^+ ions from the intracellular compartment to traverse the capillary wall and replenish Na^+ in the extracellular space (Young et al., 1987). Vascular oedema results from the degradation of the junctions between endothelial cells, transforming capillaries into 'fenestrated' capillaries, that allow extracellular (outward filtration) of proteinaceous fluid. Oncotic death of endothelial cells results in complete loss of capillary integrity and in extravassation of blood i.e. haemorrhagic conversion (Simard et al., 2007).

1.1.9 Symptoms and diagnosis

The clinical diagnosis of stroke is crucially dependent on an accurate history taken from the patient, witness or carer. The decision on whether signs and symptoms indicate a vascular event in the brain depends on many factors and includes: neurological symptoms, which are focal in stroke and negative such as loss of function, rather than positive. The onset of focal neurological symptoms is usually sudden and symptoms are often maximal at onset rather than progressive. Clinical diagnosis tends to be accurate around 80-85% of the time (Hankey, 2007).

Focal neurological symptoms arise from identifiable areas of the brain, such as unilateral weakness of the brain (cortical spinal tract), clumsiness (cerebellum), unilateral sensory loss (spinothalamic tract), speech disorder (dominant hemisphere) and double vision (ocular pathways).

1.1.9.1 Focal neurological and ocular symptoms:

Speech or language disturbances

Difficulty in:

- Understanding or expressing spoken language (dysphasia)
- Reading (dyslexia) or writing (dysgraphia)
- Calculating (dyscalculia)
- Slurred speech (dysarthria)*

Sensory symptoms

- Altered feeling on one side of the body, partly or whole (hemisensory disturbance)

- Loss of vision in one eye, partly or whole (transient monocular blindness or amaurosis fugax)
- Loss of vision in left or right half or quarter of visual field (hemianopia, quadrantanopia)
- Bilateral blindness
- Double vision (diplopia)*

Vestibular symptoms (balance)

- Spinning sensation (vertigo)*

Motor symptoms

- Weakness or clumsiness on one side of the body, partly or whole (hemiparesis)
- Bilateral weakness (paraparesis, quadriparesis)*
- Difficulty swallowing (dysphagia)*
- Imbalance (ataxia)*

Behavioural or Cognitive symptoms

- Difficulty in daily routines- dressing, brushing teeth etc
- Geographical disorientation
- Difficulty in copying diagrams such as clock, flower or intersecting cubes (visual-spatial-perceptual dysfunction)
- Forgetfulness (amnesia)*

*Does not necessarily indicate focal cerebral ischemia as an isolated symptom may be due to many other potential causes.

1.1.10 Diagnosis

1.1.10.1 Brain imaging with CT and MRI

If the clinical diagnosis is stroke, then computerised tomography (CT) brain imaging is required in order to confirm recent cerebral infarction, subarachnoid or intracerebral haemorrhage, and to exclude non-vascular intracranial pathology, for instance a brain tumour, as the cause of focal neurological symptoms and signs. Early CT brain imaging within the first few hours of stroke allows intracerebral haemorrhage to be identified straight away and in all cases. Subarachnoid haemorrhage (SAH) can be identified in about 95-97% of cases and cerebral infarction in around two thirds. The lower identified cases of cerebral infarction may be due to poor CT resolution, CT scans being too early, too small for an infarct to be imaged or because of artefact obscuring the image (particular relevant in the posterior fossa). Even within five hours, however of symptom onset, the CT scan will show abnormalities in cerebral infarction in half of cases (Von Kummer, 1997). If CT scanning is delayed for over two weeks after the onset of stroke, then CT imaging is not reliable in distinguishing ischaemic from haemorrhagic stroke. This is because CT appearances of haemorrhage will change over time to resemble the appearance of an infarct on a CT scan. Magnetic resonance imaging (MRI) however, can show intracerebral haemorrhage months and even years after the event (Hankey, 2007).

1.1.10.2 Carotid ultrasound (Doppler ultrasound)

Carotid ultrasound is a non-invasive diagnostic tool, using ultra high frequency sound waves to create images of the carotid arteries. The technique is frequently performed to detect stenosis of the carotid artery

and can also be used to measure blood-flow. It provides reliable information on localisation and extent of stenosis, as well as flow dynamics, plaque structure and vessel wall characteristics. There are limitations with the technique, including: high operator variability, inter-hospital variability, susceptibility to artefacts from calcified plaques and difficulty in distinguishing a subtotal occlusion from a total occlusion (U-King-Im et al., 2009). Also, doppler ultrasound can overestimate the severity of stenosis (Qureshi et al., 2001).

1.1.10.3 Electrocardiogram

Electrocardiogram (ECG) is a useful tool in monitoring the vital functions in patients with acute stroke and abnormal ECGs are frequently recorded in patients. The key findings looked for are atrial fibrillation (AF), myocardial infarction (MI) and arterial embolism (AE). ECG is paramount in identifying cardiac embolic stroke. Abnormalities in ECGs are frequent in acute stroke and often reflect cardiac morbidity. Also, some ECG abnormalities and increasing heart rate predict poor recovery. Common abnormalities in ECGs include: QT prolongation, ischaemic changes, arrhythmias, tachardia and U-waves (Goldstein, 1979). Patients with ischaemic stroke, atrial fibrillation, atrio-ventricular block, ST elevation and depression, or inverted T-waves have a predicted mortality at 3 months in multivalent testing (Christensen et al., 2005). ECGs have been commonly used in physiological monitoring during carotid endarterectomy (Findlay et al., 2004).

1.1.10.4 Echocardiography

A proportion of infarctions are thrombotic and the rest, as a result of emboli, are either artery to artery within the cerebral circulation or from the heart or aorta. Diagnosis of a cardiac source for emboli is based to some degree on clinical presentation and results of CT or MRI. However, diagnosis is ultimately based on ECG and echocardiography findings. Echocardiography is the technique of choice when a cardiac source of embolism is suspected (Channon K.M., 1999). The technique can either be transthoracic (non-invasive) or transoesophageal (invasive) (Chambers, 1997). Transthoracic echocardiography is useful to confirm a diagnosis when valvular heart disease is suspected. It is also valuable in documenting underlying structural heart disease. However, imaging of the aorta is limited to the proximal root and the arch in most patients. Transoesophageal imaging can image the ascending and the entire descending aorta, as well as see a clot in the left atria, also image quality is improved. The use of transoesophageal echocardiography may be required when embolic events occur in anticoagulated patients with native or prosthetic valvular heart disease, particularly if endocarditis is suspected or when transthoracic images are inconclusive (Channon K.M., 1999).

1.1.11 Acute ischaemic stroke treatments

1.1.11.1 Thrombolysis

Currently tissue plasminogen activator (t-PA) is the only thrombolytic agent licensed for use in clinical practice. However, it is thought to be most effective within the first 3 hours of ischaemic stroke onset (Jordan et al., 2007). Although, the 'European cooperative acute stroke study' (ECASS 3) trial for t-PA, demonstrated t-PA treatment 3-4.5 hours after stroke onset

can still be effective and safe (Hug et al., 2008). T-PA acts by attaching to and activating fibrin bound plasminogen, thereby mediating lysis of the thrombus and recanalisation of the artery (Hankey, 2007).

1.1.11.2 Aspirin

Aspirin is given acutely to reduce early recurrence by 15% and protects one in six patients from cardiovascular death (Albers et al., 2001). The drug prevents the aggregation of platelets by irreversibly inhibiting cyclo-oxygenase (COX) and thereby impairing thromboxine A₂ (TxA₂) synthesis in activated platelets. It also acts to limit an important positive feedback mechanism of the propagation of platelet activation during thrombosis. However, aspirin resistance is a well recognised and another antiplatelet clopidogrel, may be used for patients intolerant of aspirin. Clopidogrel blocks another feedback mechanism by irreversibly antagonising the major platelet ADP receptor P2Y. It is a pro-drug and has a marginally greater efficacy than aspirin but with an increased chance of bleeding. (Hamilton, 2009)

1.1.11.3 Stroke units

Stroke units allow specialised care and rehabilitation for stroke patients. In comparison to general wards, stroke patients receiving treatment in stroke units, have lower mortality and dependency (Fuentes and Diez-Tejedor, 2009; Langhorne et al., 1993). This is partly due to better medical management to prevent fatal complications (such as, pulmonary emboli, aspiration, pneumonia) and because geographical concentration of patients under multidisciplinary management, led by a trained consultant, focuses

on the needs of stroke patients (Stone, 1999). Stroke units are efficient, cost-effective and benefits are consistent over time. When compared with other specific stroke therapies such as aspirin, stroke units have a higher target population and greater benefit in terms of reducing mortality and dependency (Fuentes and Diez-Tejedor, 2009).

1.1.11.4 Hemicraniectomy

Space-occupying hemispheric infarctions or “Malignant” middle cerebral artery (MCA) territory infarctions are the most devastating form of ischaemic stroke. They result in poor prognosis and the case fatality rates reach 80% with conventional medical therapy (Hacke et al., 1996). It is characterised by progressive swelling of the infarct, brain tissue shifts, compartmentalised elevation of intra-cranial pressure and extension of ischaemia to adjoining vascular areas. Decompressive hemicraniectomy and duroplasty is intended to normalise intra-cranial pressure, restore compromised blood flow in the penumbra and adjacent vascular area, as well as restore the midline position of the brain stem and diencephalon (Mayer, 2007). There have recently been a number of hemicraniectomy trials for “malignant” MCA territory infarctions, including: ‘Sequential-design, multicenter, randomized, controlled trial of early decompressive craniectomy in malignant middle cerebral artery infarction’ (DECIMAL) (Vahedi et al., 2007b), ‘Decompressive surgery for the treatment of malignant infarction of the middle cerebral artery’ (DESTINY) (Juttler et al., 2007) and ‘Hemicraniectomy after middle cerebral artery infarction with life-threatening Edema trial’ (HAMLET) (Vahedi et al., 2007a). A meta-analysis of the data from the three trials, showed a substantial reduction in the risk of poor outcome and case fatality after surgery in

patients, who were randomised within 48hr from onset of stroke (Hofmeijer et al., 2009). Although hemicraniectomy improves functional outcomes, it is less effective in older patients (Curry et al., 2005).

1.1.12 Preventative ischaemic stroke treatments

1.1.12.1 Antiplatelet therapy

Antiplatelet therapies are usually recommended for patients with noncardioembolic stroke (accounts for the majority of cases of ischaemic stroke) and for long-term stroke prevention after carotid endarterectomy (Lutsep, 2009). Aspirin is one of the most widely evaluated antiplatelet medications (Albers et al., 2004) and has been used alone, or in conjunction with other antiplatelet agents such as, dipyridamole or clopidogrel in the prevention of secondary ischaemic stroke (Toyoda, 2009). Dipyridamole acts through a number of mechanisms to reduce platelet aggregation and may exert anti-inflammatory and antithrombotic actions. It inhibits the adenosine transporter, which is necessary to clear adenosine from plasma. The resulting elevations in local plasma adenosine levels stimulate adenylate cyclase. In turn, this leads to higher intracellular concentrations of cyclic adenosine monophosphate, a potent inhibitor of platelet aggregation. Prostacyclin levels are also increased by dipyridamole, stimulating NO release from the endothelium. Acting in synergy with NO, dipyridamole increases platelet cyclic guanosine monophosphate levels, which is another inhibitor of platelet aggregation (Lutsep, 2009). The use of both, aspirin and dipyridamole has a superior effect on antiplatelet aggregation compared to either alone (Muller et al., 1990). Both 'European stroke preventative study' (ESPS-2) and 'European/Australasian stroke prevention in reversible ischaemia trials'

(ESPRIT), showed aspirin plus extended-release dipyrimadamole (ER-DP) was more efficacious than aspirin alone in preventing secondary stroke (Diener et al., 1996; The ESPRIT Study Group, 2006). However, Aspirin and clopidogrel was found to be an ineffective treatment because of increased bleeding, based on results from the 'Management of atherothrombosis with Clopidogrel in High-risk' (MATCH) and 'Clopidogrel for high atherothrombotic risk and ischaemic stabilization, management, and avoidance' (CHARISMA) trials (Ruland, 2008).

1.1.12.2 Cholesterol lowering

Statin therapy has been shown to reduce the occurrence of stroke by reducing LDL cholesterol (Nassief and Marsh, 2008). The mechanism of action by statin involves inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which controls the first committed step of sterol synthesis. Niacin is effective at raising high density lipoprotein (HDL), which is associated with anti-atherosclerotic, anti-inflammatory properties and reducing cardiovascular events such as stroke. HDL also is involved in several mechanisms that protect against atherosclerosis (Sanossian et al., 2007). The use of Niacin is considered to be safe in conjunction with statins (Keener and Sanossian, 2008).

1.1.12.3 Carotid endarterectomy and stenting

Carotid endarterectomy (CEA) is a surgical procedure used as an effective means to prevent stroke by correcting severe stenosis in the carotid artery. Both the 'North American Symptomatic Carotid Endarterectomy Trial' (NASCET) (North American Symptomatic Carotid Endarterectomy

Trial Collaborators, 1991) and the 'European Carotid Surgery Trial' (ECST) (Farrell et al., 1998) shown a reduction in the recurrence of stroke after surgical treatment. Appropriate patients for CEA are those with TIA or non-disabling stroke due to 70-90% carotid stenosis; the maximum allowable operative stroke and death rate should be no more than 6%. Uncertain candidates for the procedure are those with 50-69% symptomatic stenosis and those asymptomatic with stenosis with 60% (Findlay et al., 2004). Carotid artery stenting (CAS) is beginning to emerge as an alternative technique to treat carotid stenosis. However, clinical trials including 'Stent-protected angioplasty versus carotid endarterectomy' (SPACE) (Eckstein et al., 2008), SPACE-2 (Reiff et al., 2009), 'Endarterectomy versus angioplasty in patients with symptomatic severe carotid Stenosis' (EVA-3S) (Mas et al., 2008), Carotid and vertebral artery transluminal angioplasty study (CAVATAS) (Brown et al., 2001) and 'International carotid stenting study' (ICSS) (2004) did not show better efficacy compared to CEA. Both of these techniques are best viewed as complementary rather than competing therapies and currently, it seems premature to incorporate CAS in routine practice to replace CEA (Findlay et al., 2004; Narins and Illig, 2006).

1.1.13 Sex differences in physiology outcome and treatment for stroke

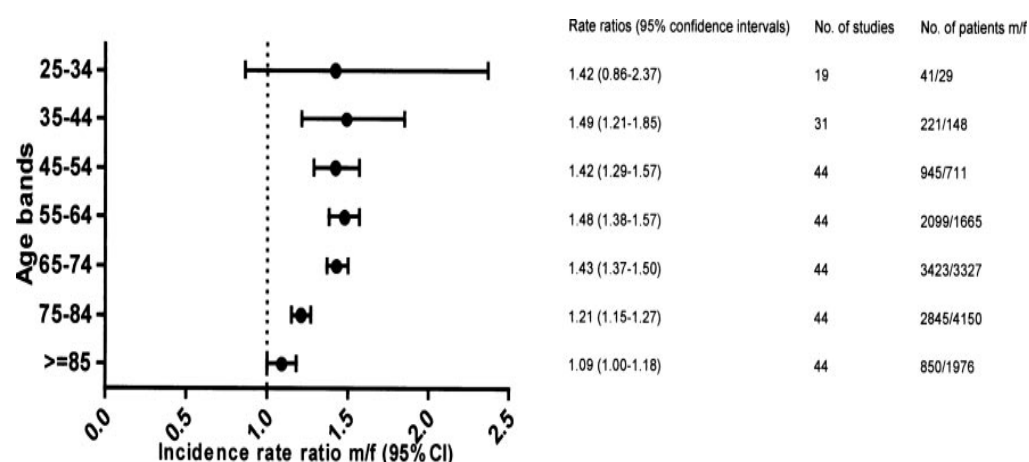
There is evidence, that differences in sex and sex steroids can influence outcome in stroke (Mehta et al., 2007). Laboratory findings have found male and female cells differ in response to death or survival signals after cerebral ischaemic injury (Vagnerova et al., 2008). Sex hormones can alter procoagulant protein levels, platelet function and also the vessel wall in a

way that may translate into sex based differences in thrombosis. The differences in platelet and coagulation functions may partly explain why women are more likely to be aspirin resistant. Also, alterations in the vessel wall biology between the two sexes could contribute to thrombosis patterns, as well as antithrombotic therapy. In the case of women, bleeding complications are higher, in part due to their smaller size and older age compared to males when given antithrombotic therapy (antiplatelets are generally given in fixed, not weight adjusted doses). Sex based differences in vessel or blood functions also contribute to increased bleeding rates in women (Bailey et al., 2009a).

1.1.14 Female sex steroids for neuroprotection

The occurrence of stroke in pre-menopausal women is significantly reduced and females have a better outcome relative to males of the same age (Barrett-Connor and Bush, 1991; Kannel and Thom, 1994). However the incidence of stroke rapidly increases as the levels of circulating sex hormones (oestrogen and progesterone) decrease around the menopause (Wenger et al., 1993) (see figure 1.2). This trend has led to the idea that female steroid hormones might be protective against stroke.

Figure 1.2: Female/Male stroke incidence rate ratios for different age bands with 95% CIs



(Appelros et al., 2009)

As a result, hormone replacement therapy (HRT) has been widely used in clinical trials, aimed at reducing the occurrence of stroke. Although, the protective role of HRT has been well documented in cardiovascular disease (Grodstein and Stampfer, 1995; Grodstein et al., 1996), its protection in stroke is less clear. A number of studies found a reduction in the occurrence of stroke after HRT (Falkeborn et al., 1993; Finucane et al., 1993; Henderson et al., 1991), while others found no significant benefit (Bushnell et al., 2001; Pedersen et al., 1997; Petitti et al., 1998; Viscoli et al., 2001). In a more recent large clinical trial, HRT was tested in the Women's Health Initiative (WHI) study, in order to assess the incidence and outcome of stroke and on other vascular events, as well as cancer. However, it was halted prematurely over concerns on increased hazards to health (Wassertheil-Smoller et al., 2003). A systematic review of completed trials involving HRT, found it was associated with elevated risk of stroke, especially of ischaemic type and of increased severity (Bath and Gray, 2005). The principle findings on stroke from the WHI clinical trials of

hormone therapy indicate that oestrogen, alone or with progestogen, increases the risk of stroke (Henderson and Lobo, 2012). Although, there was a large amount of variation between HRT trials in terms of selection criteria of participants, outcome measures, definition of an HRT user and the examination of primary and secondary incidents of stroke (Paganini-Hill, 2001). Therefore, it is difficult to conclude, definitively, the effects of HRT on stroke incidence.

Despite this set back, female sex hormones for the use of neuroprotection is still of interest and preclinical studies have shown, that oestrogen is still a candidate treatment for ischaemic stroke (Alkayed et al., 1998; Simpkins et al., 1997). Although, many of these studies, as reviewed by Stein (2001) (Stein, 2001), have focussed on protecting against ischaemic injury and hence, commenced treatment prior to injury. These studies tended to be in models of ovariectomised (Rusa et al., 1999), or reproductively ageing females (Toung et al., 2004). Preventative treatments would be beneficial, but therapies aimed at reducing damage post-stroke are essential. Any treatments concerned with reducing stroke risk should also be applied to males, who have a similar risk to post-menopausal women. Oestrogen may not be an advisable in males, due to undesirable side effects from chronic exposure. In pre-menopausal women, oestrogen administration increases the risk of uterine cancer (Grady et al., 1995) and, to counteract this, progesterone must be prescribed in combination. Hence, oestrogen only treatment would seem only applicable to a subset, albeit a significant proportion of the population (i.e. post-menopause women). However, the outcome of the oestrogen-only arm of the WHI trial was not encouraging (Powledge, 2004). The alternative sex hormone, progesterone, has been shown to have neuroprotective properties in experimental models of traumatic brain injury (TBI) (Roof et al., 1996;

Shear et al., 2002), spinal cord injury (Gonzales Deniselle et al., 2002), and stroke (Gibson and Murphy, 2004). As a treatment option, it is far more desirable for use in clinical practice. The steroid has advantages compared to oestrogen in terms of reduced side effects in males and a better safety profile, e.g. there is lower incidence of breast cancer associated with exogenous progesterone (Sharpe and Boivin, 2000).

1.2.1 Progesterone

Progesterone is a naturally occurring C21 sex steroid hormone, also known as P4 (pregn-4-ene-3, 20-dione) and is involved in the female menstrual cycle, pregnancy (supports gestation) and embryo genesis in many species including humans. Although progesterone can be produced in the lab it should not be confused with progestins, which are synthetically produced progestogens. The production of progesterone in mammals is mainly in the ovaries, particularly after ovulation in the corpus luteum and during pregnancy in the placenta. Female levels of progesterone in the menstrual cycle are relatively low at pre-ovulation phase (<2ng/ml) and then rises after ovulation (>5ng/ml). If pregnancy occurs, progesterone levels are initially maintained at luteal levels (4-20 ng/ml) but, with the onset of luteal-placenta phase, there is a shift to increase progesterone in order to support pregnancy, e.g. to reach 100-200 ng/ml at term. The concentration of progesterone drops to low levels after delivery in the placenta and during lactation. Progesterone levels are relatively low in children, postmenopausal women and in adult males, the progesterone concentration is similar to those found in women during the follicular phase of the menstrual cycle (0.2-0.8 ng/ml) (Erickson, 1995). The brain has the capability of producing progesterone and has its own mechanisms for

regulating progesterone synthesis (Corpechot et al., 1997). The brain can produce progesterone by *de novo* neurogenesis from cholesterol, via the precursor of pregnenolone. The side chain of cholesterol is cleaved by cytochrome P450_{scc}, a rate-limiting mitochondrial enzyme to form pregnanolone, which is then further converted to progesterone by 3 β -hydroxysteroid dehydrogenase (Schumacher et al., 2000).

1.2.2 Progesterone as a candidate for neuroprotection

Few effective treatments currently exist for acute ischaemic stroke. Although t-PA is effective, its use is limited by its narrow treatment window. A main focus of stroke research is the development of novel neuroprotective strategies to reduce on-going neuronal damage. Progesterone has advantages compared to oestrogen in terms of reduced side effects in males and a better safety profile, e.g. there is lower incidence of breast cancer associated with exogenous progesterone (Sharpe and Boivin, 2000). Also, no severe adverse effects have been associated with high progesterone doses in transient use (Gaver et al., 1985; Little et al., 1974). Progesterone has already been shown to be safe and effective for many other clinical applications, e.g. hormone replacement therapy and a variety of preparations are available for different modes of administration (Goletiani et al., 2007). Synthetic progestins, which are similar to progesterone but not chemically identical, can cause undesirable side effects (Simon et al., 1993). This could be a possible explanation as to why HRT studies failed since progestins were used rather than natural progesterone.

The majority of research on progesterone has been focussed on the treatment of TBI (Stein and Sayeed, 2010). Stroke has overlapping

pathophysiological mechanisms with TBI and so there is growing interest in the use of progesterone for stroke treatment. Several experimental studies have shown progesterone to be a potential neuroprotectant following cerebral ischaemia (Alkayed et al., 2000; Gibson et al., 2005b; Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002). These studies have found, post-injury administration of progesterone reduces lesion volume and improves functional outcomes in both sexes, and in both transient and permanent models of stroke. Also, studies have used 'knock-out' animals to better understand the mechanisms involved in post-injury neuroprotection by progesterone. Gibson *et al* found that, mice lacking the nitric oxide synthase-2 gene (also known as inducible nitric oxide) were unable to benefit from progesterone treatment, in reducing lesion volume (Gibson et al., 2005b).

The protective actions of progesterone appear to be numerous and have been investigated, both by *in vitro* and *in vivo* approaches. In TBI, damage caused by injury-induced lipid peroxidation was reduced by progesterone, which scavenge free radicals via a membrane-stabilising type of antioxidant action (Roof et al., 1996). This is also quite relevant in ischaemic stroke, as both reactive oxygen species and oxygen free radicals are produced during the ischaemic cascade.

Progesterone can limit excitotoxic cell death by enhancing inhibitory synaptic neurotransmitters, thereby resulting in reduced neuronal excitability. Also, it is capable of reducing excitatory amino acid release (Smith, 1991), as well as subsequent excitatory neurotransmission (Goodman et al., 1996) and enhancing GABAergic neurotransmission, as it inhibits pre-synaptic glutamate release (Majewska, 1992). Progesterone and its metabolites have vasodilatory properties and thereby increasing

blood supply to the affected ischaemic area (Molinari et al., 2001; Perusquia et al., 1996).

At the site of damage after cerebral ischaemia, inflammatory responses are generated and contribute to cell death (Leker and Shohami, 2002). Progesterone inhibits the production of pro-inflammatory cytokines and so reduce inflammation (Goodman et al., 1996). It has been observed that progesterone suppress ischaemic induced expression of interleukin (IL)-1 α , nitric oxide synthetase (NOS)-2 and transforming growth factor (TGF) β 2 and progesterone's neuroprotection is partly related to the reduction of oedema, most likely related to IL-1 β suppression (Gibson et al., 2005b). Progesterone has shown to substantially reduce the expression of tumour necrosis factor (TNF)- α , attenuate cerebral oedema and increase expression of claudin 5, which plays an important role to maintain the structure of the blood brain barrier (Jiang et al., 2009). In neuronal cultures, progesterone activates mitogen-activated protein kinase (MAPK)/Extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) signalling pathways, which are associated with neuroprotection. Neuroprotection from progesterone can also be attributed by its ability to up-regulate brain derived neurotrophic factor (BDNF), increase levels and activity of choline acetyltransferase and reduce mitochondrial dysfunction (Brinton et al., 2008). Neuroprotection has also been shown to involve the regulation of the expression of trophic factors through classical stimulation of progesterone receptors (Gonzalez SL, 2004).

Although, studies have shown post-administration of progesterone after cerebral ischaemia is neuroprotective, further research is still required to clarify our understanding. Often, researchers have used progesterone doses that have previously shown to confer neuroprotection in other studies. However, progesterone pharmacokinetics has been neglected, as

there has been no detailed pharmacokinetic study, which has measured progesterone levels in both brain and plasma over time. A pharmacokinetic study is essential in order to discover the levels of progesterone achieved and to evaluate whether other dosing methods, such as infusion, can maintain high levels of progesterone. Continuous drug administration has the advantage of avoiding the associated oscillating drug concentrations produced by intermittent bolus administration. As mentioned previously, there are numerous mechanisms which may be involved in progesterone's neuroprotective actions but much still needs to be done. It is still unclear how progesterone affects different cell types in cerebral ischaemia. Therefore, more needs to be done to investigate isolated cell types, such as neurones and microglia and how progesterone affects their functions, for instance NO production of microglia. Underlying risk factors such as, diabetes, age and hypertension can have a major influence in terms of stroke outcome. It is also possible that co-morbidities could influence the effectiveness of a neuroprotectant. However, there is a lack of studies involving co-morbidities and progesterone in experimental stroke studies. The majority have been in young, healthy and usually male animals and do not represent the demographic of stroke patients. Therefore, more studies are required to explore the effectiveness of progesterone as a neuroprotectant in these co-morbid animals, particularly the major risk factors in stroke of age, diabetes and hypertension.

1.2.3 Translational perspective on progestogens for ischaemic stroke

Progesterone has a long history of clinical use for the treatment of reproductive disorders e.g. infertility, inadequate luteal phase cycles and post-menopausal hormone replacement. However, non-reproductive related clinical applications for progesterone have been considered recently e.g. phase 3 clinical trials of 'progesterone versus placebo therapy for women with epilepsy' (Herzog et al., 2012), and 'SyNAPSe: A study of progesterone in severe traumatic brain injury' (Nelson et al., 2012). These trials can give valuable insights in the translation of experimental stroke studies to the clinic. Both trials mentioned previously, have already passed to phase-3 and have already undergone preliminary safety testing in patients, which is encouraging for other non-reproductively related clinical applications for progesterone.

The progesterone and TBI SyNAPSe trial is of particular relevance because of the use of progesterone as a neuroprotectant. Stroke and TBI have overlapping pathophysiological mechanisms, making progesterone a feasible treatment option in stroke. Patients in the SyNAPSe trial received an intravenous infusion regimen of progesterone (Nelson et al., 2012). A similar infusion method could be emulated to investigate progesterone infusion in experimental stroke work.

Also, progesterone treatment, in the progesterone for women with epilepsy trial was found to be ineffective. However, post-hoc analysis identified women with higher levels of perimenstrual seizure exacerbation were responsive to progesterone treatment (Herzog et al., 2012). This indicates progesterone treatment may have varying effectiveness in different subsets of patients. Therefore, different models may be required to

determine the effectiveness of progesterone in different subgroups if this is the case in experimental ischaemic stroke.

1.2.4 Progesterone in the brain

The uptake of progesterone (like other steroids) is via passive diffusion through the cell membrane. Progesterone acts by binding to intranuclear progesterone receptor (PR), leading to receptor conformational change, binding of PR to specific progestin response elements (PRE) in the DNA, resulting in the activation of gene expression, followed by protein synthesis. Many other co-regulatory factors are involved in these complex processes. This cascade of processes takes time, and protein synthesis can be observed after a few hours from the binding of PR, especially of two proteins, PRA and PRB. In humans both proteins are encoded by a single gene under control of distinct promoters. Each of these give rise to a distinct mRNA species that encodes for PRA or PRB (Giangrande and McDonnell, 1999). Progesterone is metabolised in several cells and in particular, the brain; progesterone metabolites unable to bind to PR but can bind to other receptors.

Progesterone can target many regions within the central nervous system (CNS) including hypothalamus, hippocampus and cortex. The receptors are broadly expressed throughout the brain and there is no apparent restriction to the any specific types of cells. However, PR expression may vary depending on brain regional areas, cell type or hormonal status (Brinton et al., 2008). Progesterone receptors are not only influenced by progesterone, but also indirectly by the sex hormone oestrogen via oestrogen receptors, which up-regulates the expression of progesterone receptors (Kastner et al., 1990). Post-mortem concentrations of

progesterone, 5 α -pregnane-3, 20-dione (5 α -DHP) and allopregnanolone in fertile and postmenopausal women vary regionally for all three steroids. Highest progesterone levels were found in the amygdala and cerebellum, while highest levels for both 5 α -DHP and allopregnanolone were observed in the substantia nigra and basal hypothalamus. The concentrations of these steroids, were found to be significantly higher in fertile women in the luteal phase as compared to postmenopausal women (Bixo et al., 1997).

Another route of signalling from progesterone is via a second mechanistic pathway that operates through non-genomic interactions. These actions in this route in general, require a rapid time frame that occur in seconds to minutes compared to genomic action, which are detected in hours to days (Gellersen et al., 2009). Other progesterone receptors unrelated to nuclear receptors have been described recently and include a number of membrane bound receptors. Both progesterone receptor membrane component (PGRMC)1 and the related PGRMC2 belong to the membrane associated receptor family, which are widespread in eukaryotes (Cahill, 2007). Other membrane progesterone receptors, which are unrelated, have been discovered. At least three sub-types of the seven transmembrane progesterone adinopectin Q receptor have been described (α , β , γ) (Thomas, 2008). These membrane progesterone receptors display high affinity, limited capacity, displaceable and specific progesterone binding. They are also coupled to G-proteins and inhibitory G proteins that down-regulate adenylyl cyclase activity (Thijssen, 2009). The transmembrane domain 7TMPR β is another receptor, which is a target for progesterone (Brinton et al., 2008). Also, the progesterone membrane binding site 25-Dx may also be involved in neuroprotection (Labombarda et al., 2003).

1.2.5 Polymorphisms in progesterone receptors and enzymes

Any polymorphisms in the PR or in enzymes, which catalyses progesterone may result in altered response. There is evidence of polymorphisms in the gene encoding for PR, and studies have already attempted to show associations between particular polymorphisms and human diseases (Diaz-Cueto et al., 2008). Presently, there is no consistent or conclusive picture, which has emerged on clinically important associations (Thijssen, 2009). The progesterone receptor gene polymorphism (PROGINS) variant occurs at frequencies of up to 0.15 in Caucasians of different origins (Romano et al., 2007). Studies indicate that, women carrying the PROGINS allele are at an increased risk of developing pathologies, which progesterone exposure is normally associated with protection, like ovarian cancer (Romano et al., 2006). However, PROGINS carriers have a reduced risk of breast cancer (Dunning et al., 1999).

The liver as a major source of metabolising enzymes, such as the hydroxylation of progesterone in the human liver by cytochrome P450 3A4 (CYP3A4) (Rendic, 2002). There are also catalysing enzymes in the brain such as CYP2D isoforms that catalyses 21-hydroxylation of allopregnanolone, progesterone and 17 α -progesterone. It is suggested that CYP2D may be involved in the regulation of endogenous neurosteroids. Polymorphism of CYP2D6 may have some influence on the nervous system through endogenous compounds, such as neuroactive steroids (Toshiro Niwa, 2004).

1.2.6 Allopregnanolone

Progesterone is converted into several metabolites such as allopregnanolone (5 α -pregnan-3 α -ol-20-one), which has been found to be

a more potent neuroprotectant compared to progesterone (Sayeed et al., 2006; Sayeed et al., 2009). The metabolite allopregnanolone, but not progesterone, has been found to strongly inhibit the mitochondrial permeability pore, which appears to be an intrinsic pathway of apoptosis-induced neuron loss (Sayeed et al., 2009). Progesterone can indirectly affect CNS function independent of PR through allopregnanolone, which acts as a positive modulator for GABA_A receptors. In a paper studying the anticonvulsant activity of progesterone and allopregnanolone in PRKO mice, it was found that PR was not required for the antiseizure effects of progesterone, which mainly occurs through its conversion to allopregnanolone (Reddy et al., 2004). This indicates that pharmacological actions of allopregnanolone may act through a different route.

Both progesterone and allopregnanolone are potent neuroprotective agents but both have different pharmacological properties. The progesterone metabolite allopregnanolone does not bind to PR, and also does not bind to the putative progesterone membrane receptor or the glucocorticoid receptor. Allopregnanolone increases GABAergic transmission, unlike progesterone, and may exert differential effects on GABA_A receptor plasticity in neurons. This is likely to account for some of the physiological actions of this compound (Biggio et al., 2006). Allopregnanolone fluctuations in the brain can also change the subunit composition of GABA_A receptors in thalamic relay neurons (Pisu et al., 2008). The divergent physiological actions of the two neurosteroids may be attributable to the conversion of progesterone to allopregnanolone, which primarily takes place in the brain and not in serum (Schumacher et al., 2007). Interestingly in a rat model of TBI, it was found that progesterone maintained procoagulants thrombin, fibrinogen and coagulation factor XIII, while allopregnanolone increased anticoagulant protein expression of tPA.

This means, it is more appropriate to use allopregnanolone in thrombotic stroke (VanLandingham et al., 2008). With this in mind, together with the knowledge that progesterone is converted to allopregnanolone in the brain, it is not known to what extent this reaction occurs before progesterone can act at various sites in the damaged brain before being converted.

1.2.7 Considerations for progesterone's mechanism for neuroprotection

As mentioned previously, progesterone can act on a variety of receptors including PR, which initiates a genomic signalling pathway and other receptors that act on non-genomic routes. Because both signalling pathways have different physiological response times after signalling, it would influence the window of opportunity for neuroprotection. If the main therapeutic response from progesterone is due to PR, then gender could be a major factor in determining the neuroprotective response. The PR is influenced by oestrogen, as mentioned previously and so varying concentrations of oestrogen present may affect the response from PR signalling. Pre-menopausal women have a greater concentration of oestrogen in their systems compared to post-menopausal females and males. There are also considerations for the transcription and metabolism of progesterone between genders and hormonal status. However, progesterone as mentioned can act via non-genomic signalling pathways. These non-genomic signalling pathways would result in a much faster physiological response. As mentioned previously, progesterone metabolites do not bind to the PR receptor and there is good indication that progesterone metabolites, such as allopregnanolone have a greater protective effect and may be the main contributor to protection (Sayeed et

al., 2006; Sayeed et al., 2009). These are some of the considerations that need to be evaluated in order to ascertain, whether progesterone will be neuroprotective in different subgroups, at what concentrations and at what therapeutic window.

1.3 Research with animal models

Treatments are usually initially tested on animals for a number of reasons. Regulatory authorities concerned with public safety require extensive animal testing to screen new treatments for toxicity and to establish safety. Animal studies provide a degree of environmental and genetic manipulation, which is rarely feasible in humans (Lemon R, 2005). If preliminary testing of treatment on animals show they are not clinically useful, then it may not be necessary to test them on humans (Hackam, 2007).

There are a variety of animal species, which have been used in the research of stroke. These range from rodents, such as mice and rats to primates like marmosets. They are a valuable tool in understanding the pathology of disease and the effects of different treatments on a living system. Cell culture or *in vitro* approaches will only give you information on the cells or tissues that are being tested. It does not however, give insight on systemic effects. This would include: pathophysiology in a systemic system, effects on different organs, tissues and cell types. A compound, which has certain effects on one type of cell, may have greatly different effects in another from different tissues in an organism. The use of animal models allows pharmacokinetics and dose response relationships to be observed for drug treatments. Also, animal models allow insightful observations on physiology and behaviour, during and after interventions. It is possible to observe the effects of pathological damage, both on the

structure of the brain and the resulting behaviour. As well as, gaining insights into the pathophysiology and causes of disease, animal studies often reveal novel targets for directed treatments.

Considerations must be made in order to select an appropriate model. These include: the breeding of animal models (if required), gestation period and time for maturity to reach desired age, behaviour of animal, the genetics of animals and strains available, the suitability of the model for experimentation, drug treatments and surgery. There are also financial considerations, not only on the actual animals themselves, but also on facilities available to keep them.

1.3.1 Choice of ischaemic model

The choice of an ischaemic model can be either global or focal. Global cerebral ischaemia occurs when blood flow to the brain is stopped or reduced, while focal cerebral ischaemia is the result of a blood clot, which has occluded a cerebral vessel and so reduces blood flow to a specific region of the brain. Ischaemic strokes tend to be focal and so the focal ischaemic model would better represent ischaemic stroke. There are a variety of focal ischaemic models available, each with their pros and cons (table 1.1).

Middle cerebral artery occlusion (MCAO) is the preferred method of inducing focal lesions that represent stroke. This is an acceptable model, as the majority of human strokes result from occlusion in the region of the middle cerebral artery (Yoo et al., 1998). Both transient and (Gibson and Murphy, 2004; Murphy et al., 2002) permanent (Ishrat et al., 2009; Jiang et al., 2009) focal cerebral ischaemia can be induced with the MCAO intraluminal filament method. The majority of human cases of stroke tend

to be permanent occlusion of cerebral arteries. However, the stroke therapeutic academic round table (STAIR) group suggests, that compounds need to show efficacy in both transient and permanent focal ischaemia models. The STAIR criteria are a set of standards for the development of pre-clinical neuroprotective drugs in order improve experimental design and reduce experimental bias. Also, the STAIR criteria suggests that, compounds which have only been shown to be only effective in pre-clinical transient ischaemic models, should only be tested in strokes where there is reperfusion, if they progress to human clinical trials (Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

Table 1.1: Advantages and disadvantages of different focal ischaemic models

Model	Advantages	Disadvantages
MCAO by permanent electrocoagulation	Good reproducibility in outcome measures achievable Successful occlusion confirmed visually by section of occlusion site Some control of infarct size and location possibly by occluding different segments of the middle cerebral artery Infarct matures quickly and is maximal within 24-48 hrs Low mortality	Requires craniectomy Technically challenging Model not suitable for transient ischaemia or thrombolysis studies
MCAO by an intraluminal filament	Good reproducibility in outcome measures achievable Suitable for transient or permanent ischaemia Technically straightforward surgery (no craniectomy)	Successful occlusion by confirmed visually Reproducibility dependent on identifying optimal filament dimensions and construction for the rodent strain and size Increased haemorrhagic risk with certain filament types Significant mortality may be encountered with large strokes (>24 hrs) and before infarct has fully evolved Model not suitable for transient ischaemia or thrombolysis studies
MCAO by endothelium-1 (topical or intraparenchymal)	Good reproducibility in outcome measures achievable Successful occlusion confirmed visually (topical model) Modifications to intraparenchymal model may allow ischaemia induction in conscious rat	Dependent on endothelium-1 vasoconstrictor potency to be consistent from batch to batch Topical administration requires craniectomy Model not suitable for thrombolysis studies
MCAO by clip/mechanical device	Successful occlusion confirmed visually Suitable for transient or permanent ischaemia	Requires craniectomy Technically challenging Likely to require more than one occlusion site, hypotension or common carotid artery occlusion to achieve reproducibility
MCAO by autologous blood clot	Most closely mimics human ischaemic stroke Model suitable for thrombolysis studies	Less reproducible than other models Less control over ischaemic location and duration More variable in outcome measures Secondary microclot formation possible with clot breakdown
MCAO by by intravascular thrombin injection	Reproducibility in cortical ischaemia and infarct volume achievable Position and persistence of clot of clot confirmed visually Model suitable for thrombolysis studies Low mortality compared to autologous clot models	Requires craniectomy Secondary microclot formation possible with clot breakdown Limited sensorimotor deficit due to small size and location of infarct
Photochemical ischaemia	Reproducibility in cortical ischaemia and infarct volume achievable Blood vessel thrombosis can be achieved through intact skull More recent modifications of the model are suitable for thrombolysis studies	Photocoagulation insult causes severe damage to affected blood vessels and substantial vasogenic oedema uncharacteristic of human stroke Limited sensorimotor deficit due to small size and location of infarct

(Macrae, 2011)

1.3.2 Choosing the species

Mice are particularly useful for experimental stroke, in terms of expense, facilities available and time it takes to reach appropriate maturation and age for experiments. Ischaemic mouse models have been increasingly used to investigate pathogenesis and treatments in stroke. This is partially due to advantages compared to rats, in newly available transgenic mice strains and specific molecular probes. Both transient and permanent MCAO models can give reproducible brain injury. Also reperfusion in the transient model is relatively easy. The type of ischaemic injury observed in a mouse model is similar to both rats and humans. Therefore, information gained from this model should be relevant (Mao et al., 2000).

1.3.3 Physiologically relevant models

Sex differences can affect the effectiveness on treatments and so it is essential to test treatments for both sexes separately. The problem of using female models is the confounder of endogenous sex steroids. Ovariectomy allows the study of the effects of oestrogen or progesterone without the confounder of endogenous sex steroids contributed by the ovary. Ovariectomised rodents have been used in stroke models and ovariectomy, results in a dramatic reduction not only in circulating oestrogen, but also progesterone. However, this procedure may result in an impairment of cognitive performance (Luine et al., 1998). The problem with ovariectomy is that, it better mimics surgically menopausal women and not necessarily the model of menopause (Singh et al., 2008). Natural menopause is characterised by lengthy continuum of biologic changes where ovarian hormone biosynthesis provides low circulating levels of sex hormones and androgens. Comparatively, surgically induced menopause

results in a severe and sudden reduction, so physiological changes occur quickly (Bachmann, 2001).

1.3.4 Limitations of using experimental stroke models

Although the mouse model is a relevant one, there are limitations to keep in mind when comparing a mouse model to human patients. Considerations are required for the brain structure between species, drug dosing and recovery.

There are a number of dissimilarities between the brains of rodents to non-human primate models and humans. As a result, there may be differences in response to an identical ischaemic insult. Mice brains are considerable smaller compared to a humans and structurally, the mouse brain surface is relatively smooth (agyric), while the human brain is more complex with many folds and fissures (gyric) (Mehra et al., 2012). Also, the human brain has a relatively larger frontal lobe compared to other brain areas. With this in mind, a model with a more similar structure to humans may be a more comparable model, such as a primate one. Primates could be used as an intermediate step for the development of a non-invasive mechanism in delivering drugs to patients. However, the use of primates can be problematic in terms of expense, facilities and time it would take to reach an appropriate age for experimental conditions, such as post-menopause. Also, concerns in terms of ethics are always associated with primate models, making approval more difficult (Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

In terms of drug dosing and recovery, it is difficult to scale up dosing regimens from rodents to humans, as well as dose and duration of drug delivery. Recovery from stroke can be far slower for humans when

compared to rodents, up to several months in fact. An optimal animal model for testing stroke recovery with drug treatments would encompass some, but not complete recovery as it would approximate human stroke (Stroke Therapy Academic Industry Roundtable (STAIR), 1999).

1.4 Clinical trials for neuroprotection

Presently the testing of neuroprotective drugs in clinical trials has resulted in disappointment, as none so far has resulted in an effective treatment in acute ischemic stroke (Green, 2002). An example would be trials that tested disufenton sodium (NXY-059) a free radical scavenger. The results for the combined 'Stroke-acute ischaemic NXY treatment' (SAINT) 1 and 2 trials for NXY-059 have resulted in no significant effect (Shuaib et al., 2007b), despite the positive pre-clinical studies, that have lead to such clinical trials. One explanation, is because of pre-clinical studies, which have inconsistent findings and more undue weight given to positive studies rather than neutral ones. A meta-analysis of available individual animal data from pre-clinical studies for NXY-059, did find it was neuroprotective. However, its efficacy may of been overstated because of bias (Bath et al., 2009).

Often in pre-clinical studies, animals are young, healthy, rarely have co-morbidities, and are not exposed to the range of competing (and interacting) interventions that humans receive. These make poor predictors of clinical outcome (Hackam, 2007). Problems of pre-clinical studies include publication bias. If there are neutral or negative studies, which are not published or are not considered, as with positive published findings, then positive studies will be over emphasised and treatments may progress to clinical trials unjustifiably. Animal studies with small sample

sizes are more likely to report higher estimates of effect than studies with larger numbers; this distortion usually regresses when all available studies are analysed in aggregate. It is not very common for animal studies to report a sample size calculation, which is a fundamental step in helping to ensure an appropriately powered precise estimate of effect (Sena et al., 2007).

The use of meta-analysis for the assessment of pre-clinical studies before the progression to clinical trials, would be an advantage, by combining results from similar studies quantitatively, to reduce biases between studies (Stewart and Parmar, 1993). In order that meta-analysis of studies is valid, statistical tools are needed in order that included studies are not biased. Tools to identify publication bias include funnel plots (plots of effect estimates against sample size) and Egger's Test (test for funnel plot asymmetry, the more asymmetric the more likely of bias) (Egger et al., 1997). Sources of biases include English language bias in which, preferential publication of negative findings of journals in other languages besides English and therefore, making locating and including of negative findings more difficult. Citation bias can also be problematic when negative studies are quoted less often, and so are more likely to be missed in searches for relevant trials. Sometimes positive trials are reported more than once in searches for trials (multiplication bias). Biases such as the ones mentioned are more likely to affect smaller studies to a greater degree, compared to much larger trials (Egger et al., 1997).

Some have argued, that the reason why clinical studies have failed after favourable preclinical studies with animals is because the animal models are inadequate for testing neuroprotective compounds (Wiebers et al., 1990). However, the biochemical mechanisms involved in cell death and functional outcome changes are very similar between the brains in humans

and animal models, such as the rat. It is more likely the design of studies maybe flawed. Randomisation, concealed allocation and blinded outcome assessment standards that are considered the norm when planning and reporting human clinical trials can be inconsistent in animal studies. In an attempt to improve the quality of studies, recommendations for standards regarding clinical neuroprotective and restorative drug development (STAIR criteria) was developed. The STAIR criteria quality uses a 8 point rating scale, a point is given for: presence of randomisation (pseudo randomised 0.5), monitoring of physiological parameters, assessment of dose response relationship, assessment of optimum time window, masked outcome measurement, assessment of outcome 1-3 days, assessment of outcomes 1-30, combined lesion volume and functional outcome (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). In addition to the STAIR criteria, blinded surgery has also been suggested (Bath et al., 2009).

At present there have only been two clinical studies that have investigated progesterone as a potential neuroprotectant. These have been in TBI and have shown improved outcome and good safety tolerance in the progesterone-group (Wright et al., 2007; Xiao et al., 2008). However, no clinical trials have yet examined the acute administration of progesterone following cerebral ischaemia so far.

1.5 AIMS

There is still a lack of understanding in progesterone's neuroprotective properties and the purpose of this project is to clarify some of these issues. The pharmacokinetics profiles in plasma and brain, following different administration methods, have not been previously investigated. Therefore,

progesterone pharmacokinetic profiles in plasma and brain, following administration via intraperitoneal (i.p.) injection, versus infusion via osmotic mini-pump with a pre-loading dose of progesterone, would be compared. The pharmacokinetic model will then be used to estimate progesterone concentrations in published studies that have shown neuroprotection. Delivering progesterone, via osmotic mini-pumps has not been employed previously and information on release characteristics could pave the way for its use in neuroprotection studies. We hypothesise that osmotic mini-pump infusion could be more effective in delivering progesterone to the target organ of the brain, when compared to a bolus intraperitoneal injection. Following this, further experiments will determine whether progesterone administration, via osmotic mini-pump, is effective at conferring neuroprotection after the onset of experimental stroke. It is hypothesised that animals receiving progesterone, via mini-pump delivery, will have a better outcome compared to vehicle treatment. Co-morbidities could influence the effectiveness of a neuroprotectant. However, there is a lack of studies involving co-morbidities and progesterone in experimental stroke studies. Therefore, the effectiveness of progesterone, as a neuroprotectant, in these co-morbid animals will be explored in the major stroke risk factor groups of aged, diabetic and hypertensive animals. We hypothesise that co-morbidity can affect the efficacy of progesterone treatment in outcomes. As well as investigating *in vivo* aspects, progesterone actions will be explored *in vitro* by studying the effects of progesterone on NO production and microglia viability, as well as the viability of SHSY5Y neuroblastoma cells, subjected to simple models of OGD that is associated with ischaemia. It is hypothesised that progesterone will reduce NO production and cell death. Furthermore, the gold-standard for assessing effects across studies within and between subgroups is to use a meta-analysis based on individual animal data (IAD).

A systematic review and meta-analysis, based on IAD of pre-clinical studies of progesterone in experimental stroke models will be conducted, in order to better understand the overall neuroprotective effectiveness of progesterone. We hypothesise meta-analysis would reveal progesterone to reduce lesion volume, but also discover other effects in different subgroups of animals.

CHAPTER 2

Progesterone pharmacokinetics in the mouse: implications for potential stroke therapy

Publications contributing to this chapter:

Wong, R., Ray, D., and Kendall, D.A. (2012). Progesterone pharmacokinetics in the mouse: implications for potential stroke therapy. *J Pharm Pharmacol* 64, 1614-1620.

2.1 INTRODUCTION

There is growing evidence to suggest that differences in gender (Appelros et al., 2009), possibly due to endogenous sex steroids, can influence the incidence and outcomes of stroke. The occurrence of stroke in pre-menopausal women is significantly lower than in men and females have better clinical outcomes relative to males of the same age (Barrett-Connor and Bush, 1991; Kannel and Thom, 1994). However, the incidence of stroke in women rapidly increases as the levels of circulating sex hormones (oestrogen and progesterone) decrease during the menopause (Wenger et al., 1993). These trends have led to the proposal that female steroid hormones might be protective against stroke, and although the majority of studies to date have focused on oestrogen (Gibson et al., 2006), attention has also been directed towards progesterone (Alkayed et al., 2000; Chen and Chopp, 1999; Gibson et al., 2005b; Gibson and Murphy, 2004; Ishrat et al., 2009; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002; Sayeed et al., 2009; Sayeed et al., 2007). Progesterone's protective properties have also been demonstrated in other models of CNS injury, including traumatic brain injury and spinal cord injury (De Nicola et al., 2009). Pre-clinical studies have shown progesterone to be neuroprotective following cerebral ischaemia (Alkayed et al., 2000; Chen and Chopp, 1999; Gibson et al., 2005b; Gibson et al., 2007; Gibson and Murphy, 2004; Ishrat et al., 2009; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002; Sayeed et al., 2009; Sayeed et al., 2007). The steroid has advantages compared to oestrogen in terms of reduced side effects in males and a better safety profile, e.g. there is lower incidence of breast cancer associated with exogenous progesterone (Sharpe and Boivin, 2000). Also, no severe adverse effects have been associated with high progesterone doses over a short course of treatment (Gaver et al., 1985;

Little et al., 1974). Progesterone has already been shown to be safe and effective for many other clinical applications, e.g. hormone replacement therapy in conjunction with oestrogen, and a variety of preparations are available for different modes of administration (Goletiani et al., 2007). In two clinical trials, progesterone, following traumatic brain injury, has been shown to be beneficial and safe (Wright et al., 2007; Xiao et al., 2008).

The neuroprotective potential of progesterone has been demonstrated in numerous experimental stroke studies and although dose-response relationships (Chen and Chopp, 1999; Kumon et al., 2000; Murphy et al., 2002; Murphy et al., 2000) and optimal time windows for administration (Jiang et al., 1996; Murphy et al., 2002; Murphy et al., 2000) have been reported, only one study has investigated progesterone concentrations in both brain and plasma following administration (Coomber and Gibson, 2010). In that study, the progesterone concentration was measured 7 days after progesterone pellet implantation. It is important to determine the pharmacokinetic profile in normal healthy animals first, to determine if there are any toxic effects to a range of doses, without the complication of disease present. Once this is determined, drugs can then be tested in disease models. In terms of neuroprotection, the time immediately after the onset of stroke is the most crucial in terms of efficacy for neuroprotectants (Ginsberg, 2008). Therefore, it is crucial to establish progesterone's pharmacokinetic profile immediately following cerebral ischaemia and for the following period of 1-2 days. The ideal solution would be to achieve therapeutic concentrations in the brain quickly and for these to be sustained over the critical period of time. Continuous drug administration has the advantage of avoiding the associated oscillating drug concentrations produced by intermittent bolus administration.

The aim of these experiments described in this chapter was to compare progesterone pharmacokinetic profiles in plasma and brain following administration via intraperitoneal (i.p.) injection, versus infusion via osmotic mini-pump with a pre-loading dose of progesterone. The pharmacokinetic model was then used to estimate progesterone concentrations in published studies that have shown neuroprotection. Delivering progesterone via osmotic mini-pumps has not been employed previously and information about the release characteristics could pave the way for its use in neuroprotection studies.

2.2 MATERIALS AND METHODS

2.2.1. Ether extraction protocol

Brains were homogenised with ultrapure water to make a 20% w/v homogenate and underwent ether extraction based on a protocol mentioned previously. 5 ml of ice-cold diethyl ether was added to brain homogenates in glass test tubes, vortexed and left for 15 minutes. Test tubes were then placed in an acetone and dry ice bath for 30 seconds or until the water/debris layer at the bottom was frozen. The ether layer was then decanted into a new test tube and the procedure was repeated 3 times. The new test tube containing the extracted sample was then placed into the acetone and dry ice bath to freeze any residual water and the ether layer was decanted into a scintillation vial. This was placed in a fume cupboard and allowed to evaporate to dryness.

2.2.2 Animals

Experiments were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986 (Project License 40/3207) and were approved by the University of Nottingham ethical review process. Male C57 Bl/6 mice (10-12 weeks) were used, weighing between 20.5 and 30.5g at the time of injection. Mice were assigned a code by a non-experimenter and assigned randomly to different time point groups. In total, 74 animals were used in this study, 3 at time point 0 for both i.p. injection alone and infusion, 58 animals for i.p. only (see table 2.1 for individual numbers at each time point) and 13 for infusion only (see figure 2.2 for individual numbers at each time point).

2.2.3 Progesterone administration

Mice were injected intraperitoneally with 8 mg/Kg of progesterone (USP) (Sigma, St Louise, MO, U.S.A) using a solution of 16mg/ml progesterone dissolved in 100% dimethyl sulfoxide (DMSO; Sigma). Control animals only received 100% DMSO. Mice were injected under isoflurane anaesthesia (induction 4%: maintenance 1.5% in a NO₂/O₂ 70/30% mixture) and were allowed to recover from the anaesthesia. Animals receiving i.p. injection without mini-pump implantation also received anaesthesia for control.

Mini-pumps with an infusion rate of 1.0 µl/h and a reservoir with up to 3 days delivery capacity (Alzet 1003D; Alzet, Cupertino, CA, USA) were loaded with progesterone solution (50mg/ml dissolved at 37°C in 100% DMSO). These were submerged in 0.9% sterile saline solution at 37°C overnight in order to prime the pumps to start progesterone delivery immediately after implantation. Mini-pumps were implanted subcutaneously behind the neck under isoflurane anaesthesia (induction

4%: maintenance 1.5% in a NO₂/O₂ 70/30% mixture). Mice were given a progesterone loading dose (8mg/Kg i.p. progesterone dissolved in 100% DMSO) immediately after implantation before being allowed to recover from anaesthesia.

2.2.4 Determination of progesterone in plasma and brain

At appropriate time points i.e. 4h, 6h and 24h, following the onset of dosing mice were given an i.p. overdose of pentobarbital and killed by exsanguination via cardiac puncture. Blood samples were collected from the cardiac puncture and whole brains were removed. The brains collected at 4 and 24 h were dissected into 6 regions: vulnerable cortex, non-vulnerable cortex, hippocampus and striatum, midbrain, cerebellum and brainstem for progesterone assay. The vulnerable cortex is the area of the cortex closest to the middle cerebral artery (MCA), which is occluded in the majority of studies (for example see Gibson & Murphy (Gibson and Murphy, 2004)) to surgically induce stroke, and therefore, suffers the greatest degree of ischaemic damage (dissection of this region was carried out 2.5 mm anterior and posterior from MCA). Non-vulnerable cortex comprises the anterior and posterior regions of the cortex furthest away from the middle cerebral artery (beyond 2.5 mm anterior and posterior from MCA) and, would therefore be least affected in future studies utilising experimental stroke via occlusion of the MCA.

Blood was centrifuged at 16,000g, at room temperature for 15 minutes and plasma collected. Plasma and brains were stored at -80°C for later analysis. Brains were homogenised with ultrapure water to make a 20% w/v homogenate and underwent ether extraction based on the protocol described previously (section 2.2.1). Ether extracts were stored at -80°C

and then reconstituted with 5% ethanol in ultrapure water prior to progesterone assay using a commercial kit (Progesterone Enzyme Immunoassay Kit; Cayman Chemicals, Michigan, USA). Progesterone standards were also ether extracted and recovery was calculated to be 98.9%. Samples were analysed in duplicate according to the manufacturer's instructions and absorbance was read using a plate reader (Dynex Technologies MRX II) at 405nm.

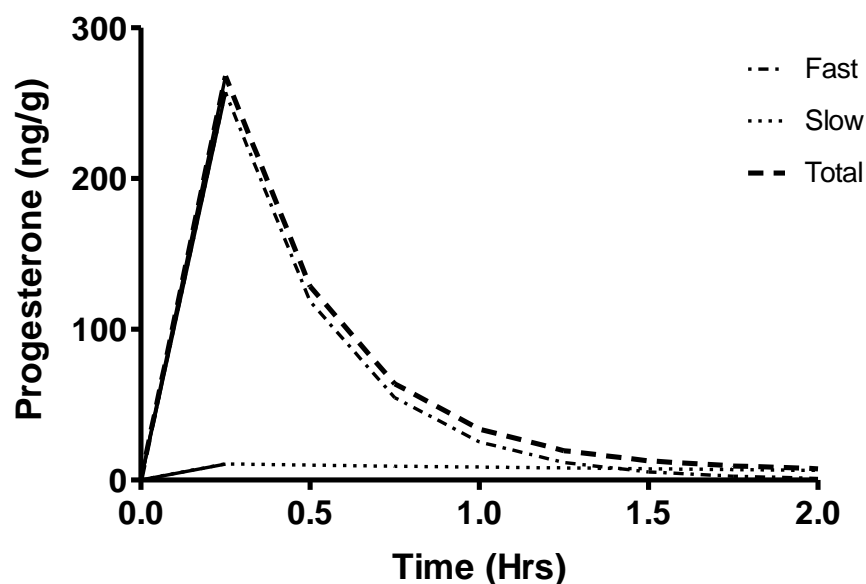
2.2.5 Data analysis

Data are expressed as means \pm standard error of the means (SEM). Differences between plasma and brain progesterone concentration time points were analysed with a linear mixed effects model (PASW Statistics 18.0.2, IBM, Chicago, IL, USA). Area under the curve (AUC) was measured using the linear trapezoidal rule, which was used to calculate total body clearance and apparent volume of distribution (Vengpedersen, 1989). GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to analyse differences in brain regional progesterone concentrations at different time points, using two-way analysis of variance (ANOVA). If a significant difference was observed, post-hoc Bonferroni multiple comparison was performed. The criterion for statistical significance was $P < 0.05$ for all analyses. Pharmacokinetic parameters for components were calculated by methods and equations given by Gibaldi and Pierrier (Gibaldi, 1982) and Martin (Martin, 1986), using Excel and GraphPad Prism Version 5.0 for windows.

2.3 RESULTS

Inspection of the progesterone absorption, distribution, elimination profile after a single bolus injection revealed two phases and so a two-compartment analytical model was applied. The two-compartment model assumes that, following drug administration into the central compartment, the drug distributes between that compartment and the peripheral compartment. This distribution i.e. equilibration is not instantaneous between the compartments and in a concentration time-profile a biphasic response can be seen. Mean values of plasma and brain progesterone concentration are shown in Table 2.1. These values were used to construct a time-course, which shows total progesterone concentration over time and comprises a fast and slow component after one bolus i.p. injection dose of 8mg/kg of progesterone in both plasma and brain (see figure 2.1 for brain profile).

Figure 2.1: Elimination of progesterone from the brain after one bolus intraperitoneal dose of 8mg/kg.



The total component comprises both fast and slow components. Fast component half-life was 0.2 hr with 96% eliminated for this component and the slow component being 2.3 hr. There were no samples taken prior to the first 15 minute sampling point; therefore, the extrapolation from the time zero to the first time point is a theoretical estimate (shown in solid lines). This figure shows only the first 2 hrs of elimination and the sampling intervals at this time were 0, 0.25, 0.5, 1 and 2 hrs.

In plasma the fast component half-life of progesterone was 0.2 hr with 96% eliminated and 23.1 hr for the slow component. In brain, the fast component half-life for progesterone was 0.2 hr with 96% eliminated and the slow component half-life was 2.3 hr. Half-life calculations were based on sampling time points of 15 minutes onwards. Therefore, half-life calculations may not represent the true half-life of progesterone because earlier measurements prior 15 minutes were not available for half-life

calculations. The maximum concentration time (T max) after one bolus injection was estimated to be in the region of up to 15 minutes for both plasma and brain. The maximum recorded concentration (C max) was achieved at this time point for both brain and plasma (plasma; 110.3 ng/ml, brain; 268.3 ng/g). Similarly, the AUC was 297.6 ng/hr/ml in plasma, and 172.8 ng/hr/g in brain (for a time out to 24 hrs). The difference between plasma and brain progesterone concentrations at sampling time points was statistically significant ($P<0.001$) (Table 2.1).

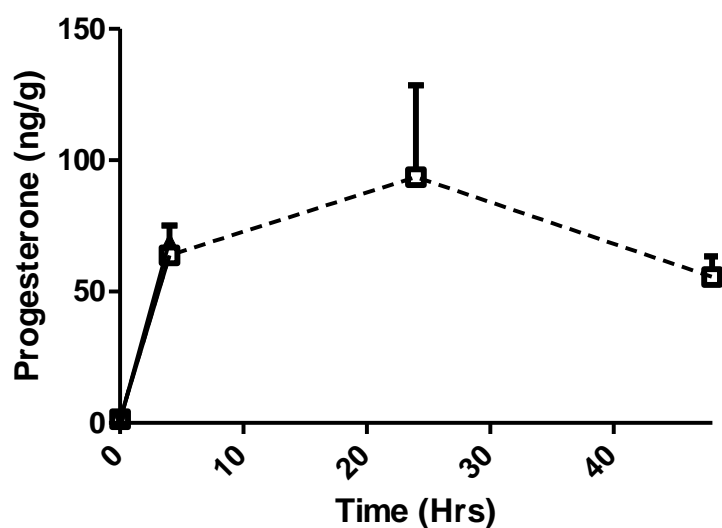
Table 2.1: Progesterone concentrations in plasma and brain, following a single bolus i.p. dose of 8mg/kg.

Time (hrs)	n	Mean Plasma (ng/ml)	Mean Brain (ng/g)	Mean of Brain/Plasma Ratios
0	3	0.89 ± 0.41	0.72 ± 0.56	1.58 ± 1.29
0.25	7	110.28 ± 40.69	268.27 ± 136.49	1.74 ± 0.38
0.5	9	65.58 ± 23.62	66.38 ± 15.59	1.48 ± 0.29
1	7	34.32 ± 18.71	10.35 ± 3.64	0.61 ± 0.14
2	7	13.94 ± 3.93	13.91 ± 5.41	0.87 ± 0.22
4	7	11.81 ± 1.37	2.48 ± 0.34	0.21 ± 0.02
8	7	16.80 ± 1.85	4.65 ± 0.89	0.29 ± 0.05
16	7	5.07 ± 1.27	1.23 ± 0.20	0.61 ± 0.35
24	7	7.63 ± 1.73	1.73 ± 0.72	0.35 ± 0.15

Data are expressed as means ± SEM. Mean brain plasma ratios are the overall means of brain ng/g divided by plasma ng/ml. Differences between plasma and brain were significant ($P<0.001$).

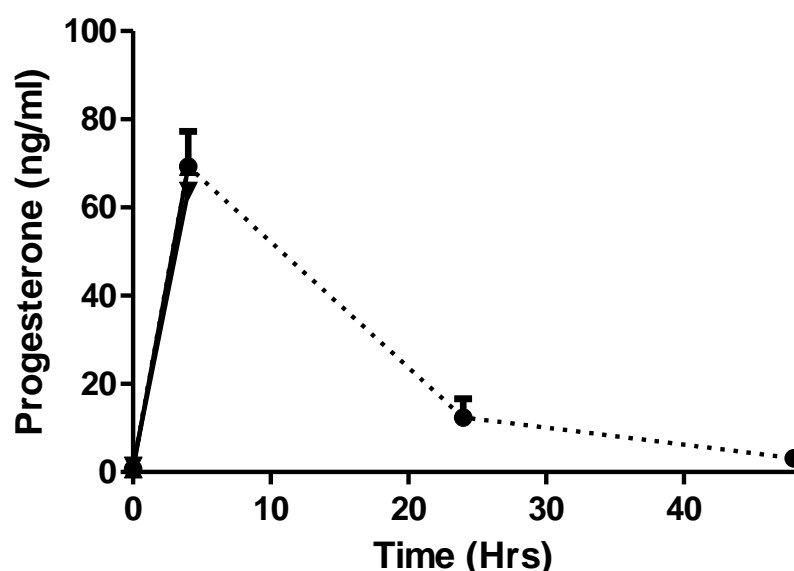
Progesterone concentrations achieved by a loading i.p. dose and mini-pump infusion were much higher compared to the bolus dose alone at the same time points. T max was 4 hr in plasma and 24 hr in brain. C max for plasma was 69.3 ng/ml and 87.5 ng/ml for brain. The AUC for plasma was 968.0 ng/hr/ml up to 24 hrs and 1167.0 ng/hr/ml up to 48 hrs while brain reached 1641.8 ng/hr/g up to 24hrs and 3358.6 ng/hr/g up to 48hrs. Brain progesterone concentrations stayed at a relatively high steady state concentration, peaking at 24 hrs (figure 2.2). In comparison, progesterone plasma concentrations did not stay at steady state concentrations but was in decline after peaking at 15 minutes (figure 2.3). The AUC shows that far higher progesterone concentrations were reached and maintained in both plasma and brain using osmotic mini-pump delivery with loading dose compared to a bolus dose alone.

Figure 2.2: Brain progesterone concentrations in mini-pump-implanted male C57 Bl/6 mice injected with 8mg/kg of progesterone after implantation.



Data are expressed as means \pm SEM. There were no samples taken prior to 4 hrs; therefore the extrapolation from time zero to the first time point is a theoretical estimate from time 0 concentration (shown in solid lines). n=3-5 (times 0 hrs n=3, 4 hrs n= 4, 24 hrs n= 5 and 48 hrs n=4) independent samples analysed in duplicate.

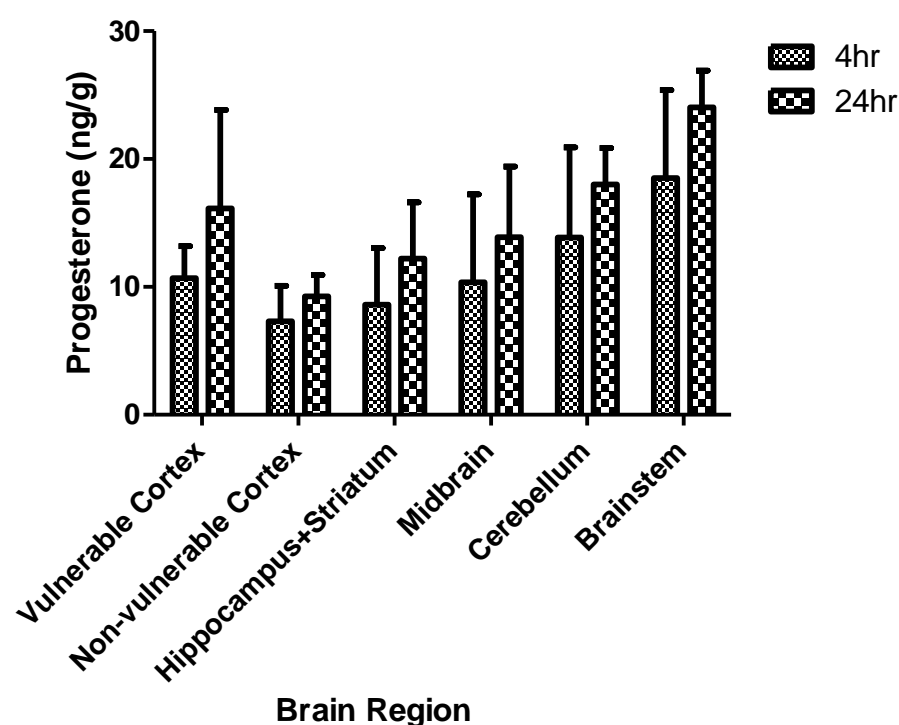
Figure 2.3: Plasma progesterone concentrations in mini-pump-implanted male C57 BI/6 mice injected with 8mg/kg of progesterone after implantation.



Data are expressed as means \pm SEM. There were no samples taken prior to 4 hrs; therefore the extrapolation from time zero to the first time point is a theoretical estimate from time 0 concentration (shown in solid lines). $n=3-5$ (times 0 hrs $n=3$, 4 hrs $n=4$, 24 hrs $n=5$ and 48 hrs $n=4$) independent samples analysed in duplicate.

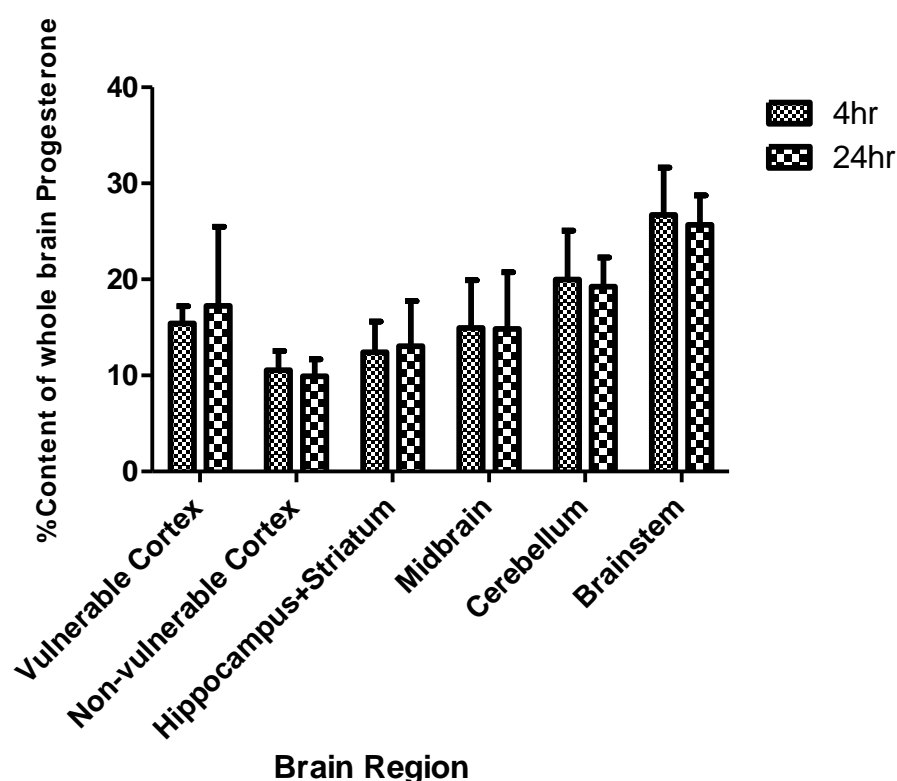
The difference between plasma and brain progesterone concentrations was found to be significant in mini-pump-implanted animals ($P<0.001$). Progesterone regional concentrations were found to be elevated at 24hrs, compared to 4hrs [$F(1,36)=2.92$, $P=0.0258$] (figure 2.4), but the distribution of progesterone between brain regions was found to be unchanged [$F(5,42)=2.17$, $P=0.0756$] (figure 2.5). This indicates regional distribution of progesterone to be in a steady state, unchanged by progesterone concentration in the whole brain.

Figure 2.4: Progesterone concentration in difference brain regions after mini-pump implantation



Progesterone regional concentrations were found to be elevated at 24hrs, compared to 4hrs [$F(1,36)= 2.92$, $P=0.0258$]. Regional concentrations of progesterone (ng/g) were measured at 4 hrs ($n=4$ per region) and 24 hrs ($n=5$ per region) after loading dose of 8mg/kg and mini-pump implantation. Brains were dissected into 6 regions: vulnerable cortex, non-vulnerable cortex, hippocampus and striatum, midbrain, cerebellum and brainstem for progesterone assay. The vulnerable cortex is the area of the cortex closest to the MCA. Non-vulnerable cortex comprises the anterior and posterior regions of the cortex furthest away from the MCA and, would therefore be least affected in future studies utilising experimental stroke via occlusion of the MCA. Data are expressed as means \pm SEM.

Figure 2.5: Distribution of progesterone in whole brain after mini-pump implantation



The distribution of progesterone between brain regions was found to be unchanged [$F(5,42) = 2.17$, $P = 0.0756$]. Regional progesterone distribution were measured at 4 hrs ($n = 4$ per region) and 24 hrs ($n = 5$ per region) after loading dose of 8mg/kg and mini-pump implantation. Brains were dissected into 6 regions: vulnerable cortex, non-vulnerable cortex, hippocampus and striatum, midbrain, cerebellum and brainstem for progesterone assay. The vulnerable cortex is the area of the cortex closest to the MCA. Non-vulnerable cortex comprises the anterior and posterior regions of the cortex furthest away from the MCA and, would therefore be least affected in future studies utilising experimental stroke via occlusion of the MCA. Data are expressed as means \pm SEM.

2.4 DISCUSSION

Before attempts can be made to optimise the dosing of animals in preclinical experiments, the pharmacokinetics of progesterone need to be established. The study by Jiang *et al.* conducted an experimental stroke study, using a dosing regimen based on other studies (Roof *et al.*, 1996; Roof *et al.*, 1992), which had shown neuroprotection and they conducted their own preliminary pharmacokinetic experiment (Jiang *et al.*, 1996). In that preliminary pharmacokinetic study ($n=2$), they found plasma progesterone concentrations to rise to 41.9 and 70.7 ng/ml 4hrs after i.p. administration of 4 mg/kg progesterone dissolved in DMSO, from pre-injection levels of 7.17 and 5.29 ng/ml in male rats. However, the progesterone half-life in this and other studies was not calculated, although the metabolism and clearance of progesterone is believed to be quite rapid in mice, Beckley *et al.* found after a single i.p. injection of 5mg/kg progesterone in mice, plasma progesterone dropped from around 125 ng/ml (0.4 μ M) at 0.5 hrs to around 30 ng/ml (0.1 μ M) at 2 hrs and below 20 ng/ml at 8 hrs (<0.1 μ M), but did not calculate the progesterone half-life or investigated other forms of dosing (Beckley and Finn, 2007). The use of mini-pumps for the delivery of agents has been used previously in pre-clinical stroke studies (Altura and Altura, 1999; Farr *et al.*, 2007; Marshall *et al.*, 2000), but progesterone dosing, via mini-pump in pre-clinical studies of stroke have not been conducted previously. Hence, due to the lack of detailed pharmacokinetic information for both i.p. and infusion of progesterone, it was necessary to establish a pharmacokinetic profile in the mouse model to allow the construction of a dosing regimen to maintain a therapeutic level of progesterone.

Previous studies demonstrating the neuroprotective properties of progesterone following experimental stroke in male mice, such as that by

Gibson & Murphy (Gibson and Murphy, 2004), have used a dosing regimen of 8mg/kg progesterone injected intraperitoneally (dissolved in 100% DMSO). This dose and route of administration were also found to be neuroprotective in several other studies (Gibson et al., 2005b; Gibson et al., 2011; Ishrat et al., 2009; Kumon et al., 2000; Sayeed et al., 2009; Sayeed et al., 2007). The present study was focused on clarifying the progesterone pharmacokinetic profile in both plasma and brain, comparing intraperitoneal administration previously shown to be neuroprotective, to an infusion delivery method of progesterone. Infusion methods are commonly used to maintain drug concentrations within the therapeutic range. Osmotic mini-pumps were used as the method of infusion as they release progesterone at a constant rate, therefore avoiding the diminishing levels of release over time as is the case with pellet implants. However, with mini-pumps there is an unavoidable delay before effective tissue concentrations are reached, so an i.p. loading dose of progesterone was employed to achieve effective concentrations rapidly. Progesterone was only measured up to 48 hrs from the start of treatment due to brain levels peaking and reaching steady state by this time.

Progesterone delivered via a single i.p. injection had a very short half-life in both plasma and brain, with brain progesterone levels reaching more than twice those in plasma at 15 minutes. It is not surprising that progesterone accumulates in the brain, since steroid hormones are very lipophilic and so have a tendency to move from plasma into fatty tissues (Zhu et al., 2004). Progesterone is readily able to cross the blood-brain and blood-nerve barriers, and rapidly accumulates in neural tissue (Pardridge and Mietus, 1979; Schumacher et al., 2000). In this study, after 30 minutes, progesterone levels in the brain fell to concentrations similar to plasma and eventually dropped below plasma progesterone levels (Table

2.1) which is not surprising given that progesterone undergoes metabolism in the brain (Billiar et al., 1975; Frye et al., 1998; Johnson et al., 1976). A major pathway responsible for the metabolism of progesterone is via the 5 α -reductase enzyme, which is widespread in both neurons and glial cells, with neurons displaying higher rates of enzyme activity compared to glial cells. In particular, white matter structures contain very high levels of the enzyme. Progesterone is converted to 5 α -dihydroprogesterone and is further metabolised to other compounds (Lephart et al., 2001). One such metabolite, allopregnanolone (5 α ,3 α -pregnanolone) may convey greater neuroprotection in ischaemic stroke compared to progesterone (Sayeed et al., 2006). Progesterone has a high metabolic clearance rate and about 75% of metabolism takes place in the liver and spleen (Lobo, 2000). This may partly explain why the plasma progesterone level is lower in comparison to the brain concentrations of animals with mini-pump infusions, due to rapid splenic and hepatic elimination. It is unclear why plasma progesterone concentrations are higher than brain at later time points but there may be some release of progesterone from fatty tissue reservoirs which have accumulated the steroid.

Application of the i.p. progesterone pharmacokinetic model derived in the present study to the results of Gibson & Murphy (Gibson and Murphy, 2004) predict that progesterone concentrations in the brain would peak transiently at about 250 ng/g (0.8 μ M) around 15 mins after injection with almost total clearance within 2 hrs. Despite delivering only transient peaks of the steroid in the brain, this dosing method was successful in providing neuroprotection to young male mice (Gibson and Murphy, 2004). However, the ideal profile would be to maintain progesterone concentration at as high as possible concentrations due to the lack of toxicity from the steroid (Gaver et al., 1985; Little et al., 1974). Progesterone treatment seems to

be most effective 0-2 hrs following cerebral ischaemia (Gibson et al., 2007), although no studies in pre-clinical stroke have administered progesterone after this period. Therefore, an early, high transient peak of progesterone may be enough to initiate neuroprotection, although future studies will need to determine whether the higher maintained progesterone concentrations achieved by infusion produce better neuroprotection after experimental stroke. Due to the transient nature of the concentration peak following a single i.p. dose, a repeated i.p. dosing schedule was not assessed.

In comparison to physiological levels in mice, plasma progesterone concentrations reached 110.28 ng/ml (0.35 μ M) 15 minutes after bolus injection, which is far higher compared to the healthy male mice that received no progesterone at zero time point (0.89 ng/ml or 0.003 μ M) (Table 2.9). This plasma concentration is somewhat higher than that measured around the peak of pregnancy. McCormac & Greenwald found the peak progesterone concentration during pregnancy to reach 39 ng/ml (0.12 μ M) in plasma (McCormac.Jt and Greenwal.Gs, 1974) and Virgo & Bellward found serum progesterone levels in pregnant mice to peak at 81.9 ng/ml (0.26 μ M) (Virgo B.B., 1974). Brain progesterone concentrations in the first 30 minutes after dosing were above the highest progesterone concentration found in the brain during the mouse oestrous cycle (approximately ~45 ng/g or ~0.14) in meta-oestrus 2 (Corpechot et al., 1997), whilst levels in the brains of mini-pump implanted mice remained relatively constant between about 50 and 100 ng/g (0.16 μ M and 0.32 μ M) from 4-48 hrs, approximating to high physiological levels. The oestrous cycle has been shown to affect the severity of focal cerebral ischaemia in pre-clinical studies. In pro-oestrous spontaneous hypertensive rats, the infarct after focal cerebral ischaemia was found to be smaller compared to

animals in meta-oestrus (Carswell et al., 2000), meta-oestrous spontaneous hypertensive rats also had larger infarcts than respective males that underwent ischaemia during meta-oestrus (Carswell et al., 1999).

There are few reported studies involving progesterone and experimental stroke using infusion delivery; Alkayed *et al* (Alkayed et al., 2000) is the only published study utilising an infusion method with rats and Coomber & Gibson (Coomber and Gibson, 2010) with mice. The Coomber & Gibson (Coomber and Gibson, 2010) study is the only one to date that has measured progesterone in both plasma and brain after treatment with slow releasing tablets containing 50 mg of progesterone inserted subcutaneously 7 days prior to middle cerebral artery occlusion (MCAO). That study failed to show benefit in terms of lesion volume, but the progesterone concentration measured in plasma and brain on the day that animals received MCAO was found to be 11.6 ng/ml in plasma and 38.2 ng/g in brain, well below the steady-state levels measured in the present study. Therefore, it would be worthwhile employing the present mini-pump infusion approach in order to achieve higher maintained brain levels in stroke models. However, increasing progesterone dose does not necessarily result in greater neuroprotection. Some studies have used higher doses of progesterone via i.p. injections but found no significant beneficial effect, ranging from 10-32 mg/kg (Chen and Chopp, 1999; Murphy et al., 2002; Murphy et al., 2000). Chen *et al* found 8 mg/kg to be neuroprotective but not 4 mg/kg or 32 mg/kg (Chen and Chopp, 1999), indicating a dose-response efficacy window. Lower doses of progesterone may not be enough to reach pharmacological concentrations, while other mechanisms may be involved in terms of higher doses. Cutler *et al* used 16mg/kg of progesterone and found acute withdrawal increases apoptosis,

inflammation and anxiety behaviours during the acute recovery phase after TBI compared to tapered withdrawal, but regardless of progesterone treatment regimen all progesterone treated animals showed improvement over vehicle treatment (Cutler et al., 2005). They postulated that acute withdrawal leads to sudden decrease in GABA-A interactions with the progesterone metabolite allopregnanolone, resulting in increased NMDA activation, which eventually leads to a excitatory neural environment. Under the added stress of trauma, this effect is amplified and results in excitotoxicity but with gradual withdrawal, this excitotoxicity, secondary injury and inflammation are not exacerbated (Cutler et al., 2006b). Because both TBI and stroke share similar pathological mechanisms, it is possible that high 32 mg/kg progesterone i.p. dose used in Chen *et al* may amplify this acute withdrawal effect in stroke. The high dose of 32 mg/kg in Chen *et al* did reduce lesion volume but was not significant compared to 8mg/kg dose. Therefore, tapered withdrawal with high doses of progesterone is worth considering. As there are very few published studies involving infusion methods and all have been given pre-emptively before surgically induced stroke (Alkayed et al., 2000; Coomber and Gibson, 2010), further investigation is needed to determine whether this infusion method is effective when used immediately after stroke.

There was no difference in the accumulation of progesterone in various brain areas (Figure 2.4). Progesterone appears to distribute itself equally in areas of predominant white and gray matter. In ischaemic stroke, both white and gray matter, are vulnerable to damage and so potential neuroprotectants should ideally access and protect both compartments (Falcao et al., 2004). Therefore, it is encouraging that progesterone is able to accumulate in both compartments since the lack of white matter protection may be a contributing factor in the failure of experimental

neuroprotective strategies (Cheng et al., 2004; Ho et al., 2005). However, caution is needed when extrapolating results from rodent models to humans as the amount of white matter in rodents is far smaller in comparison (Green, 2003).

In conclusion, this study demonstrates that osmotic mini-pump delivery (in conjunction with a bolus i.p loading dose) is an effective way of delivering progesterone to the target areas of the brain. Future studies will employ this delivery method to assess potential neuroprotection in experimental stroke.

CHAPTER 3

Effects of progesterone and oxygen glucose deprivation on microglial and neuroblastoma cells

3.1 INTRODUCTION

Ischaemic stroke involves complex excitotoxic, inflammatory, and vascular mechanisms that lead to neuronal cell death. Inflammatory cells and mediators are important contributing factors in ischaemic brain injury (del Zoppo et al., 2000). Stroke triggers an inflammatory reaction that is initiated hours after stroke onset and plays a central role in the pathogenesis of the condition (Son et al., 2009). This inflammatory response is characterised by the accumulation of inflammatory cells, including blood-derived leukocytes and activation and accumulation of microglia within the brain tissue, which subsequently leads to brain injury (Jiang et al., 2009). The disruption of the blood-brain barrier (BBB) after stroke allows blood-derived leukocytes to enter the brain, further amplifying inflammatory signal cascades (Han and Yenari, 2003).

Microglia cells are the resident macrophages of the brain and function as scavenger cells in the event of infection, trauma, neurodegeneration, inflammation and ischaemia (El Khoury et al., 1998; Thomas, 1992). Once activated, microglia undergo morphological transformation into phagocytes, making them indistinguishable from circulating macrophages (Wang et al., 2007). There is considerable evidence to suggest that microglial activation precedes and predominates over macrophage infiltration after cerebral ischaemia (Jin et al., 2010). Active microglia, are particularly detrimental to the injured brain and contribute to infarct volume (Schilling et al., 2003; Schilling et al., 2005). Inflammatory cells can infiltrate around the ischaemic area and several pro-inflammatory genes or mediators are strongly expressed in the ischaemic brain (Stoll et al., 1998). These expressed mediators include; inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines, such as interleukin (iL)-6 and tumour necrosis factor (TNF)- α

(Kim et al., 2002). As well as having a detrimental role in cerebral ischaemia, microglia have a potentially positive role in the resolution of inflammation and are sources of growth factors for repair, including insulin-like growth factor 1 (IGF-1) and glial cell line-derived neurotrophic factor (GDNF) (Iadecola and Anrather, 2011).

Substantial evidence supports the involvement of nitric oxide (NO) in ischaemic brain injury (Moro et al., 2004). Immediately after the start of ischaemia, vasodilatory effects of NO produced, mainly by endothelial NOS (eNOS) protects the brain by limiting the degree of flow reduction caused by the arterial occlusion. However, after the development of ischaemia, NO produced by neuronal nitric oxide synthase (nNOS), followed by inducible iNOS, contributes to the development of brain injury (del Zoppo et al., 2000). Under basal conditions, NO release from microglial cells is negligible but stimulation with lipopolysaccharide (LPS) or cytokines such as TNF- α and interferon gamma (IFN γ) causes microglial cells to release substantial amounts of free radical NO (Kawahara et al., 2009; Weinstein et al., 2008).

Progesterone has been shown to act at multiple sites and through various pathways to either slow or interrupt the complex processes involved in neuronal cell death. Mechanisms of neuroprotection include; preventing accumulation of fluid inside neurons and astrocytes, up-regulation of GABA (counteracting glutamate-mediated excitotoxicity), antioxidant effects, reduction of inflammatory cytokines and decreased apoptosis (Stein et al., 2008). The iNOS gene is transcriptionally activated, probably in response to the surge of proinflammatory cytokines such as interleukin (IL)-1 β and TNF- α following cerebral ischaemia (Murphy et al., 2000). Progesterone has been shown to suppress the inflammatory response and iNOS expression following cerebral ischaemia *in vivo* (Gibson et al., 2005b).

Also, it has been observed that progesterone decreases iNOS mRNA expression and iNOS promoter activity in macrophages *in vitro* (Lieb et al., 2003). The expression and function of iNOS can be reduced by progesterone or via its activators, including IL-1 β and TNF- α (Coughlan et al., 2005).

There are key aspects in which *in vitro* models differ from *in vivo* models. Typically, a longer duration of an anoxic or hypoxic insult is required to kill neurons *in vitro*, adenosine triphosphate (ATP) depletion is less severe and the release of glutamate is delayed compared to ischaemia *in vivo* (del Zoppo et al., 2007). The absence of blood vessels and blood flow *in vitro* eliminates important structural and functional components of the damage process found *in vivo*, including the infiltration of inflammatory cells (Taoufik and Probert, 2008). Also, the composition and responsiveness of glial cells *in vitro* differs from those in intact brain (Yuan, 2009). Despite these differences, there are still similarities in the way isolated cells behave under substrate stress and the way in which cells behave under the catastrophic conditions of stroke *in vivo* (Arumugam et al., 2007). The use of *in vitro* cell models allows stroke mechanisms to be investigated without interference from conflicting processes that are present *in vivo*. *In vitro* models can provide simple, highly controlled experimental systems, which can generate detailed basic information as to how cell types respond to oxygen and glucose deprivation (OGD). However, the complexity of the architecture of the brain also requires *in vivo* models of ischaemic stroke (Woodruff et al., 2011). Therefore, both *in vitro* and *in vivo* approaches can complement one another in stroke research.

The aim of the present chapter is to investigate the effects of progesterone on NO production, microglial viability and the viability of SHSY5Y

neuroblastoma cells, subjected to simple models of OGD representative of cerebral ischaemia.

3.2 MATERIALS AND METHODS

3.2.1 Maintaining SHSY5Y and BV-2/HAPI cell culture stocks

BV-2 microglial cells were a generous gift from Prof. Nephi Stella, University of Washington, while SHSY5Y and highly aggressively proliferating immortalised (HAPI) cells were a generous gift from Prof. Andrew Bennett, University of Nottingham. The two microglia cells lines (BV-2 and HAPI) cells were utilised, in case of differences in inflammatory response e.g. HAPI cells could be considered to model more closely to activated cells. Cell stocks were grown in 75-cm² culture flasks containing 20ml of DMEM (Dulbecco's Modified Eagle Medium) culture medium with 10% foetal bovine serum (FBS) and 2mM L-glutamine. This media was used unless otherwise stated for growing cells and in the initial seeding of plates for experiments. Cells in culture flasks were left to grow in the incubator at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity until they were sub-confluent $\sim 3 \times 10^6$ cells/ml (to prevent ischaemic conditioning) and were then sub-cultured to maintain cell stocks or for experiments. The cells were sub-cultured by dissociating cells with 1 x trypsin-EDTA (ethylenediaminetetraacetic acid) and split to a dilution of 1:5 for SHSY5Y and 1:10 for BV-2/HAPI to maintain cell stocks.

3.2.2 LPS experiments

BV-2 cells were sub-cultured from cell stocks for use in experiments and were seeded onto 24 well plates (1:10 from cell stock). Each well contained 1 ml of DMEM growth media (previously described for maintaining cell stocks) and the cells were left to grow until confluent at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity. Media was then aspirated and replaced with serum-free media (SFM) containing 0.01% DMSO with either or combination of control, 100ng/ml lipopolysaccharide (LPS), progesterone (5-50 µM), mifepristone (10 µM), depending on experimental variables and placed in an incubator at 37°C with 5% CO₂ and 95% air (v/v), at 90% humidity for 24hrs before neutral red viability testing and nitric oxide quantification (NO). Progesterone and Mifepristone stock concentrations were 1000x greater than the final required concentration, dissolved in 100% DMSO. Stock concentrations were then diluted 1:1000 with the required media to attain final concentration at 0.01% DMSO. LPS stocks and final dilution are accomplished in the same manner except LPS stock was dissolved in phosphate-buffered saline (PBS).

3.2.3 OGD experiments

3.2.3.1 Cell Preparation

When preparing cells for ischaemic experiments, cells stocks were sub-cultured using DMEM growth media and seeded at 1×10^5 cells/ml onto 96-well culture plates. Each well contained 200µl total volume per well. Cultures were then incubated at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity for 48hrs to be used in experiments relating to ischaemia.

3.2.3.2 OGD and controls

When plates were ready, culture medium was aspirated from wells and washed twice with glucose-free deoxygenated SFM (200 µl for each well per wash) for cells undergoing ischaemia or SFM for non-ischaemic wells. Glucose-free DMEM was deoxygenated by bubbling 5% CO₂ and 95% N₂ (v/v) through the medium for 15 minutes. After the wash stage, glucose-free deoxygenated DMEM containing 0.1% DMSO with a combination of vehicle (0.1% DMSO only), progesterone (0.1-100 µM) or mifepristone (10 µM), depending on experimental design, was added to wells (100 µl total volume per well) designated for the anaerobic chamber. Oxygen concentration in the chamber was maintained at <1 % throughout the incubation period using a ProOx 110 controller and chamber (BioSpherix). Plates were then placed in an anaerobic chamber that was flushed with 5% CO₂ and 95% N₂ (v/v). Cell cultures were kept in the chamber for between 2 and 8 hrs at 37°C and at 90% humidity. For cells not designated for the anaerobic chamber; after the wash stage, SFM-containing 0.1% DMSO with a combination of control, progesterone (10-100 µM) or mifepristone (10 µM), depending on experimental design, was added to the wells (100 µl total volume per well). These cells were then placed at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity during the incubation period and these cells were referred to be in 'normoxic conditions'. Progesterone and Mifepristone stock dilutions and final concentrations were achieved in the same manner as described in LPS experiments.

3.2.3.3 Re-oxygenation

Once the ischaemic period was finished, the deoxygenated media was aspirated off and replaced with SFM containing different concentrations of

progesterone in 0.1% DMSO (w/v) or 0.1% DMSO vehicle for the re-oxygenating treatment back to normal conditions at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity for 24hrs. Cells designated for 'normal conditions' were treated in the same manner.

3.2.3.4 Assessment of cell viability

Viability assays determine the ability of cells to maintain or recover their viability. Each type of assay assesses a particular cell function i.e. MTT and resazurin assays are based on reduction of colouring reagents by enzymes in the mitochondria to determine mitochondrial function; neutral red is based on the cells ability to incorporate and bind the dye to lysosomes, and trypan blue assesses cell membrane integrity. Therefore, a combination of viability assays can indicate which particular cell functions may be involved in cell death.

3.2.3.5 Resazurin viability assay

Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is used as an oxidation-reduction indicator in cell viability assays. It is a blue dye, itself non-fluorescent until it is reduced in mitochondria by oxidoreductase, to the pink coloured, and highly red fluorescent resorufin. In the presence of diaphorase as the enzyme, NADH or NADPH is the reductant that converts resazurin to resorufin. Therefore, resazurin/diaphorase/NADPH system can be used to detect NADH, NADPH, or diaphorase level and any biochemical or enzyme activity, which is involved in a biochemical reaction generating NADH or NADPH. Resazurin is both non-toxic and stable in culture medium,

as well as being simple to measure continuously in a high throughput manner (Zhang et al., 2004).

Media was aspirated and wells were washed with the same volume of PBS or media in the well. 100 μ M of resazurin in Hank's balanced salt solution (HBSS) (w/v) was added to a volume of 200 μ l in each well. Cells were then incubated in normal conditions of 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity for 45 minutes. Plates were then read using a plate reader at 540 nm, immediately after incubation. The viability was calculated from % of control in normoxic conditions.

3.2.3.6 MTT viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay measures the activity of mitochondrial dehydrogenase, that reduces MTT tetrazolium bromide dye to purple formazan in living cells. Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes, mostly in the cytosolic compartment of the cell. The reduction of MTT and other tetrazolium dyes increase with cellular metabolic activity due to elevated NAD(P)H flux. A solubilisation solution is added to dissolve the insoluble formazan product to a coloured solution. The absorbance of the coloured solution can then be read and used to quantify cells.

Media was aspirated from 96 well plates and washed twice with PBS. MTT was added to DMEM growth medium (see cell culture) to a concentration of 50 μ M then MTT in media (200 μ l) was added to each well. Cells were incubated at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity for 2 hrs. Media containing MTT was aspirated and an equal volume of propan-2-ol was added to each well. This was then mixed by pipetting up and down

several times to dissolve the formazan product from MTT metabolism. Wells were then read at 620 nm in a plate reader (Dynex Technologies MRX II). The viability was calculated from % of control in normal conditions.

3.2.3.7 Trypan blue with automated cell-counter viability assay

Trypan blue is a vital dye, which selectively colours dead cells blue. The chromophore is negatively charged and does not interact with the cell unless the membrane is damaged and the membrane potential collapsed. Cells that exclude the dye are, therefore, viable (Freshney, 1987). A TC10 automated cell counter (Bio-Rad, Hemel Hempstead, UK) was also used to assess cell viability with trypan blue.

Media from 96 well plates was aspirated and 20 µl of trypsin-EDTA was added to each well and incubated at 37°C for 5 minutes. SFM was then added to each well (180 µl) and the well contents were mixed by pipetting up and down several times to dislodge adherent cells from the surface and from other cells to make a homogenous cell suspension. The cell suspension was mixed with equal parts of trypan blue solution (0.4%, Bio-Rad, Hemel Hempstead, UK) and 10 µl of cell suspension/trypan blue solution was pipette onto cell counter slides. Slides were then placed into the cell counter to read the cell viability. Viability was measured as % control and measured in duplicate.

3.2.3.8 Neutral red viability assay

The neutral red viability assay is based on the ability of viable cells to incorporate and bind the dye, neutral red. This weakly cationic dye

penetrates cell membranes by non-ionic passive diffusion and concentrates into the liposomes. The dye is then extracted from viable cells using an acidified ethanol solution and the absorbance of the solubilised dye is quantified using a spectrophotometer. It is, therefore, possible to distinguish between viable, damaged or dead cells according to their specific lysosomal capacity for taking up the dye (Repetto et al., 2008).

Media was aspirated and wells washed with PBS (1ml per wash) before adding 400 µl per well of SFM containing 100 µg/ml of neutral red. Cells were incubated for 1 hr at 37°C with 5% CO₂ and 95% air (v/v) at 90% humidity. After incubation, neutral red media was aspirated and wells washed 2x with PBS (1 ml per wash) before adding 200 µl of fixative solution (50% ethanol, 1% glacial acetic acid in distilled water). This was incubated as before for 1 hr and read using a plate reader at 540nm (Dynex Technologies MRX II). Viability was calculated as % difference from control in normal conditions.

3.2.3.9 Nitric oxide measurements

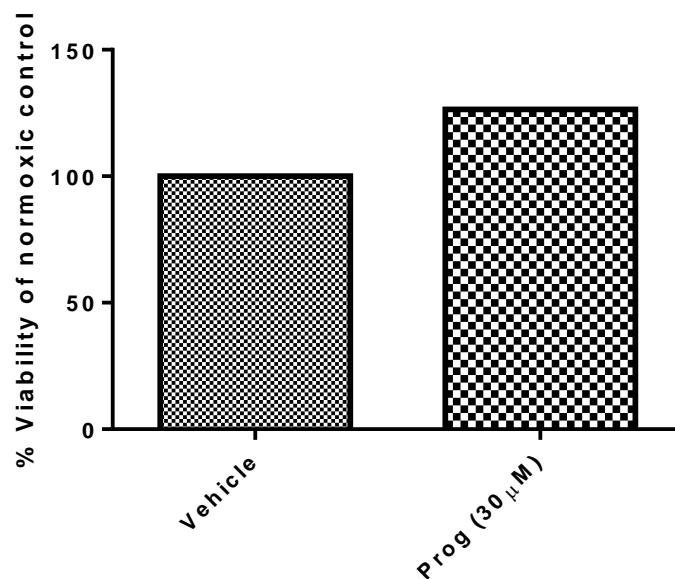
Nitric oxide (NO) was measured using the Greiss reaction, which detects the presence of organic nitrite compounds. Culture medium (100 µl) was transferred into each well of a 96 well plate, each containing 100 µl of Greiss reagent. Greiss reagent consists of 20 mg/ml (w/v) of sulphanilamide and 1 mg/ml (w/v) of N-1-napthyethylene dihydrochloride dissolved in 1M hydrochloric acid (HCL). Standards were made using NaNO₂ dissolved in media (100 µl) with Greiss reagent (100 µl) to construct a calibration graph to quantify absolute NO in sample media. Plates were read with a plate reader at 540nm (Dynex Technologies MRX II).

3.3 RESULTS

3.3.1 Nitric oxide production and viability of BV-2 microglia in OGD conditions

BV-2 microglial cells were exposed to progesterone in normoxic conditions for 4 hrs and did not reduce viability (figure 3.1).

Figure 3.1: BV-2 viability after 4 hrs in normoxic conditions

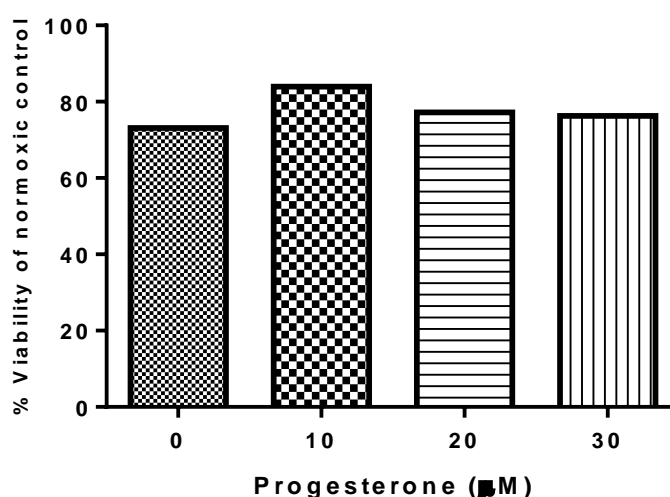


Cell viability is expressed as % of normoxic control (untreated cells). Vehicle and progesterone (prog, 30 μM) cultures were incubated in normoxic conditions for 4hrs and cell viability was measured using the Neutral Red assay. Data are expressed as means of 4 repeats on one occasion.

Cells were then exposed to OGD conditions and their effect on the production of NO determined. Increased NO production has been demonstrated in OGD conditions (Lu et al., 2006; Sun et al., 2005). However, in the present study, OGD conditions were not found to enhance

NO production above background in any experiments. The first experiment employed 4 hrs of OGD and NO production was measured directly after the OGD period. At this stage it was thought that the length of OGD might not be long enough to induce significant levels of NO formation; OGD control viability was 73.1% (figure 3.2) and cells may not have had adequate time to produce significant amounts of NO at 4 hrs ($0.88 \pm 0.33 \mu\text{M}$).

Figure 3.2: BV-2 viability after 4 hrs in OGD

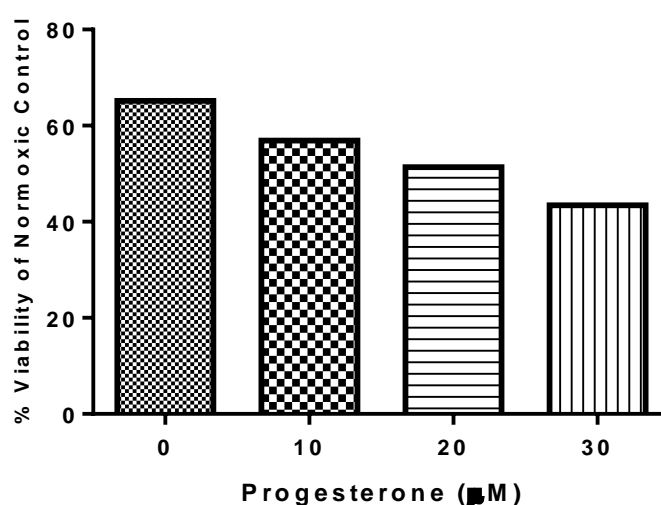


Cell viability is expressed as % of normoxic control (untreated cells). Cultures were incubated for 4 hrs with increasing progesterone concentrations (0-30 μM) and cell viability was measured using Neutral Red assay. All treatment wells contained 0.1% DMSO. Data are expressed as means of 4 repeats on one occasion.

Therefore, the time of incubation was extended in further experiments. Park *et al* found re-oxygenation was required to stimulate NO production after hypoxia (Park *et al.*, 2002). However, longer OGD periods of 6 hrs

and 8 hrs with 24 hrs of re-oxygenation (0.84 ± 0.28 and $0.85 \pm 0.28 \mu\text{M}$ respectively) were not effective at producing NO above background ($1.54 \pm 0.89 \mu\text{M}$). At 6 hrs with re-oxygenation, OGD conditions reduced viability to 65.15% compared with normoxic control. The addition of progesterone further reduced viability compared to untreated cells in OGD (figure 3.3).

Figure 3.3: BV-2 viability after 6hrs treatment in OGD followed by 24 hrs re-oxygenation

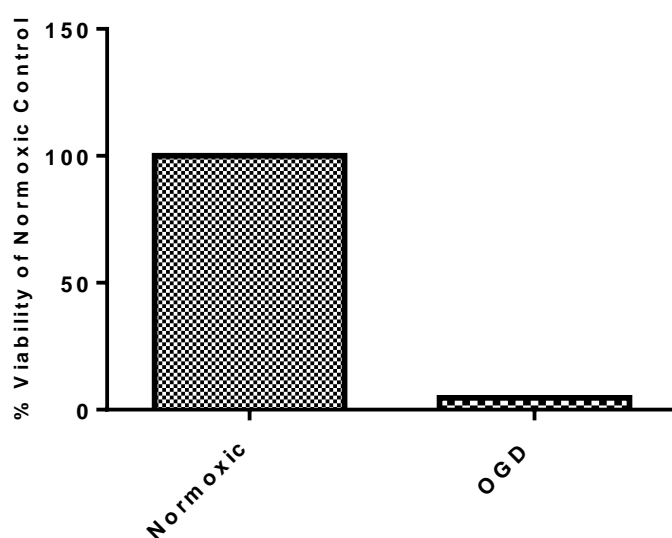


Cell viability is expressed as % of normoxic control. Cultures were incubated with increasing concentrations of progesterone (P0-30 μM) in OGD conditions, followed by 24 hrs of reoxygenation in SFM. All treatment wells contained 0.1% DMSO. Cell viability was then measured with Neutral Red assay. Data are expressed as means of eight repeats in one occasion.

After 8 hrs of OGD, followed by re-oxygenation, viability of OGD control was severely reduced, compared to normoxic conditions ($4.67\% \pm 0.64$, compared to normoxic control) and therefore, was not an appropriate time

point to use to evaluate the effects of progesterone on NO production (figure 3.4).

Figure 3.4: BV-2 viability after 8 hrs of OGD followed by 24 hrs of re-oxygenation

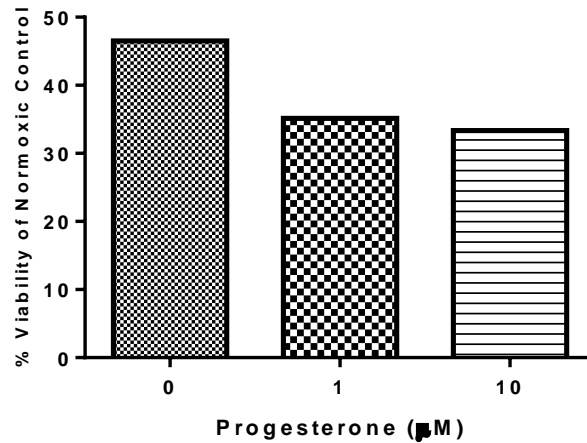


Cell viability is expressed as % of cells in normoxic conditions. Cell cultures were incubated for 8hrs either in normal glucose and oxygen (normoxic) or oxygen and glucose deprived (OGD) conditions, followed by 24 hrs of reoxygenation in SFM. All treatment wells contained 0.1% DMSO. Cell viability was then measured using Neutral Red assay. Data are expressed as means of eight repeats on one occasion.

3.3.2 Nitric oxide production and viability of HAPI microglia in OGD conditions

BV-2 Cell viability was reduced by OGD conditions, although this did not increase NO production. Therefore, a different microglial cell line of highly aggressively proliferating immortalised (HAPI) cells were utilised, in order to observe if OGD conditions can increase NO production in this model of activated microglia. HAPI microglial cells were exposed to OGD conditions to ascertain whether NO was produced. The length of exposure was 6 hrs with 24 hrs re-oxygenation, as this was shown, in earlier experiments, to produce significant differences in viability at this time with the BV-2 microglia. However, HAPI cells did not produce NO above background concentrations found in normoxic conditions (9.11 μM) when exposed to OGD (8.98 μM). Background NO concentrations in HAPI cells (9.11 μM) were greater compared to BV-2 (1.54 μM). HAPI cell viability was similar to that of BV-2 cells undergoing OGD. HAPI cells exposed to OGD had reduced viability compared to normoxic controls and progesterone treatment reduced viability to OGD control in the same condition (figure 3.5).

Figure 3.5: HAPI viability after 6hrs treatment in OGD followed by 24 hrs re- oxygenation



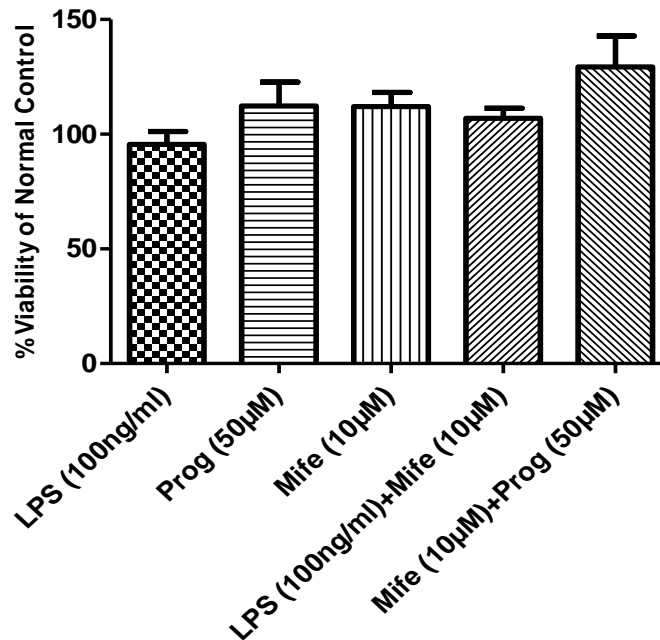
Cell viability is expressed as % of normoxic control. Cultures were incubated with increasing concentrations of progesterone (0-10µM) in OGD conditions, followed by 24hrs of re-oxygenation in SFM. All treatment wells contained 0.1% DMSO. Cell viability was then measured with the Neutral Red assay. Data are expressed as means of eight repeats on one occasion.

3.3.3 Nitric oxide production and viability of BV-2 microglia in the presence of LPS

Bacterial lipopolysaccharide (LPS) has been used frequently to study the release of nitric oxide in microglia, (for example see: (Horvath et al., 2008; Jiang et al., 2011; Son et al., 2009)). Nitric oxide (NO) production was measured using the Greiss assay to determine nitrite and viability was measured with the Neutral Red assay in BV-2 microglia. Viability of cells was not significantly affected by LPS (figure 3.6), but LPS significantly enhanced the production of NO in BV-2 cells, in the presence ($P<0.01$) or

absence of the progesterone receptor (PR) antagonist mifepristone ($P < 0.01$).

Figure 3.6: Viability of BV-2 controls

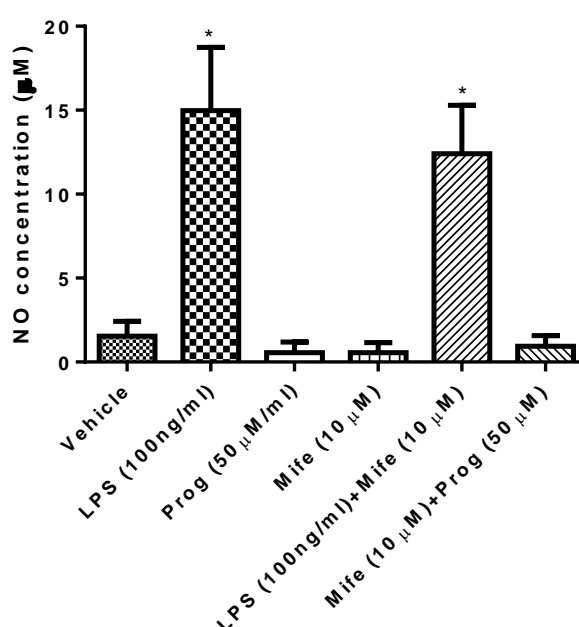


BV-2 cells were treated with Lipopolysaccharide (LPS, 100ng/ml), progesterone (Prog, 50µM) and progesterone receptor antagonist mifepristone (Mife, 10 µM). All treatment wells contained 0.1% DMSO. After 24 hr incubation in normoxic conditions, cell viability was measured with Neutral Red assay. Viability is expressed as % of normal control (untreated cells). Data are expressed as means \pm SEM ($n=3$). No significance was found between groups in cell viability using one-way ANOVA [$F(5,12)= 2.20$, $P= 0.1219$].

Analysis of controls found a significant difference between groups [$F(5,12)=10.92$, $P=0.0004$]. Newman-Keuls post-hoc analysis revealed the addition of progesterone, mifepristone or a combination of the two, did not

affect NO production in the absence of LPS (figure 3.7). These results indicate that LPS is required to induce NO production and that this is not controlled by endogenous progesterone receptor activity. Later experiments will determine the effects of progesterone on viability and NO production in the presence of LPS and whether the addition of mifepristone will have further affects.

Figure 3.7: Nitric oxide production of BV-2 controls



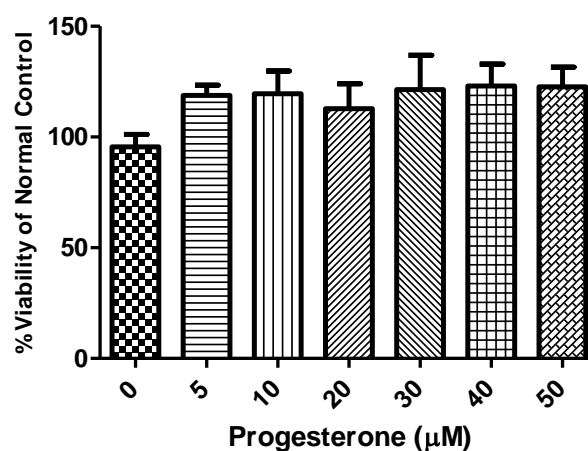
NO production using the Greiss assay after 24 hr incubation for control groups. Control groups include normal cell culture media (normal control), lipopolysaccharide (LPS, 100ng/ml), progesterone (Prog, 100µM) and progesterone antagonist mifepristone (Mife, 10µM). All treatment wells contained 0.1% DMSO. One-way ANOVA reveals a significant difference in groups [$F(5,12)=10.92$, $P=0.0004$] and Newman-Keuls post-hoc analysis reveals the LPS group and LPS + Mife group to be significantly different

from other controls ($P < 0.01^*$). Data are expressed as means \pm SEM ($n=3$).

3.3.4 Nitric oxide production and viability of BV-2 microglia in the presence of LPS, progesterone and mifepristone

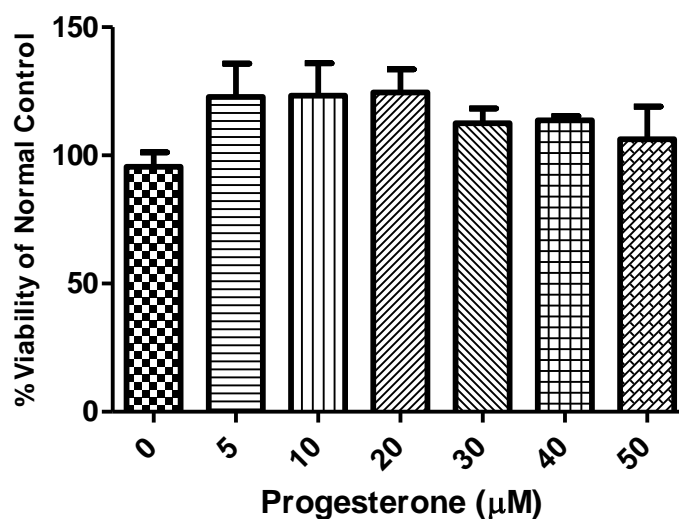
There was no effect of progesterone on BV2 viability with [$F(6,14) = 0.96$, $P = 0.4877$] or without mifepristone [$F(6,14) = 1.23$, $P = 0.3507$] (figures 3.8 and 3.9). However, increasing concentrations of progesterone significantly decreased LPS-induced production of NO and the presence of mifepristone did not alter this [$F(1,28) = 1.71$, $P = 0.2014$] (figure 3.10). This indicates that the PR receptor is not necessarily needed in the attenuation of NO production by progesterone. Also, because cell viability was unaffected by progesterone, the effect of the steroid on NO production is not simply due to reduced cell viability.

Figure 3.8: Viability of BV-2 cells in the presence of LPS and increasing concentrations of progesterone



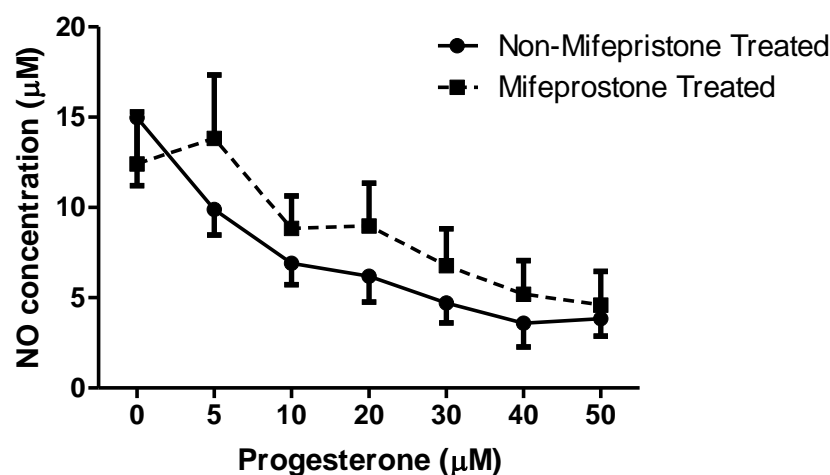
Cell viability is expressed as % of normal control exposed to normoxic cell culture media. Cultures were exposed to lipopolysaccharide (LPS, 100ng/ml) and increasing concentrations of progesterone (Prog, 0-50µM). All treatment wells contained 0.1% DMSO. After 24 hr incubation in normoxic conditions, cell viability was measured with the Neutral Red assay. Data are expressed as means \pm SEM (n=3). No significant differences were found between groups regarding cell viability using one-way ANOVA [$F(6,14)= 0.96$, $P=0.4877$].

Figure 3.9: Viability of BV-2 cells in the presence of LPS plus mifepristone and treated with increasing concentrations of progesterone



Cell viability is expressed as % of normal control exposed to normoxic cell culture media. Cultures were exposed to lipopolysaccharide (LPS, 100ng/ml) plus mifepristone (Mife, 10 µM) and increasing concentrations of progesterone (Prog, 0-50µM). All treatment wells contained 0.1% DMSO. After 24 hr incubation in normoxic conditions, cell viability was measured with Neutral Red assay. Data are expressed as means \pm SEM (n=3). No significant differences were found between groups regarding cell viability using one-way ANOVA [$F(6,14)= 1.23$, $P= 0.3507$].

Figure 3.10: Nitric oxide production in BV-2 microglial cells exposed to LPS with and without mifepristone and treated with increasing concentrations of progesterone



NO production using the Greiss Reagent assay after 24hr incubation. Cells were exposed to LPS, (100ng/ml) with or without mifepristone (10 µM) and increasing concentrations of progesterone in normoxic conditions. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM (n=3). Two-way ANOVA revealed increasing concentrations of progesterone significantly decreases NO production [$F(6,28)=5.812$, $P=0.0005$]. However, there was no significant difference in NO production, when mifepristone was present [$F(1,28)= 1.71$, $P= 0.2014$].

3.3.5 SHSY5Y neuronal cell line viability analysis with trypan blue (cell counter) viability assay

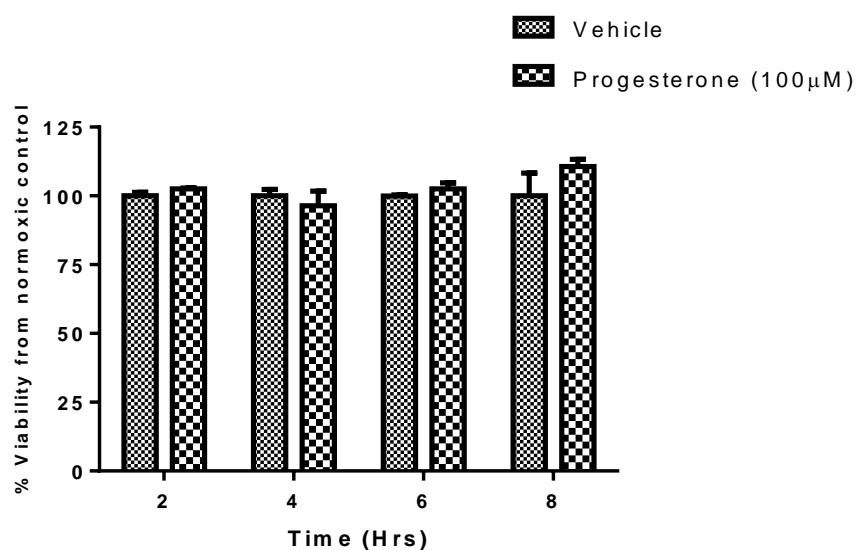
OGD reduced microglial cell viability, although this appears to be unrelated to NO production due to earlier experiments showing microglial cell viability to be unchanged by LPS. The presence of progesterone was observed to enhance rather than to reverse the impact of OGD.

Interestingly, progesterone did reduce microglial cell function, as reflected by the reduced NO production in response to LPS.

It therefore appears that any protective effects of progesterone that are apparent *in vivo* are not reflected by changes in microglial viability *in vitro*, and that progesterone might provide direct protection for neurones challenged by ischaemia. Thus, progesterone treatment and OGD conditions were then applied to human neuronal cell lines. Initially experiments were undertaken to optimise viability assays for SHSY5Y neuronal cell lines (see method development 3.2.4), before it was decided to use trypan blue with automated cell counting to monitor SHSY5Y cell viability.

The viability of SHSY5Y control (untreated) and progesterone (100 μ M)-treated cells was analysed over increasing incubation periods in normoxic conditions. Although, in previous experiments involving microglia, the maximum concentration of progesterone used was 50 μ M, progesterone concentrations up to 100 μ M were used in these experiments to observe greater pronounced differences. There were no differences found between control and progesterone treatment [$F(1,18) = 0.64$, $P = 0.4341$] (figure 3.11).

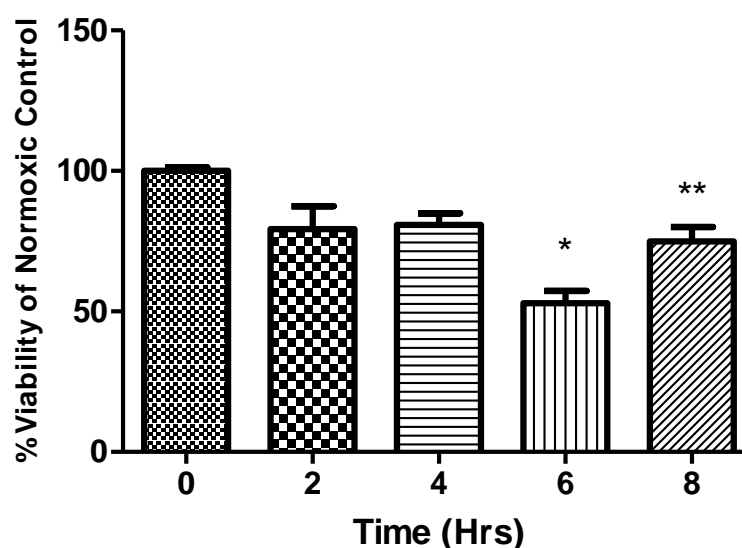
Figure 3.11: SHSY5Y viability of vehicle and progesterone in normoxic conditions over time using the trypan blue assay



Cell viability is expressed as % of normoxic control. Cultures consisted of vehicle and progesterone (100 μM)-treated cells, exposed to normoxic conditions. All treatment wells contained 0.1% DMSO. Cell viability was measured with the trypan blue assay. Data are expressed as means \pm SEM ($n= 3-5$ for each group). Analysis with two-way ANOVA shows there was no significant difference between control and progesterone treatment in normoxic conditions [$F(1,18)= 0.64$, $P= 0.4341$].

After establishing progesterone to have no effect on viability in normoxic conditions, cells were then introduced to increasing OGD conditions, to determine the incubation period required to significantly reduce viability. At 6 hrs or more of OGD, cell viability was significantly reduced (figure 3.12).

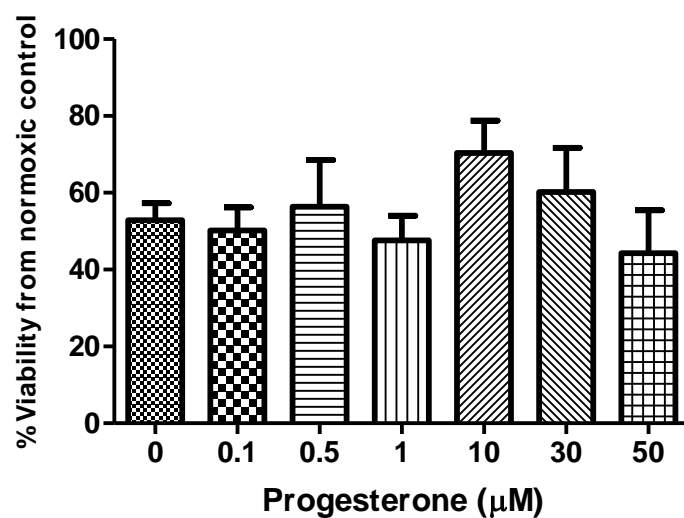
Figure 3.12: SHSY5Y viability in OGD conditions over time using the trypan blue assay



Cell viability is expressed as % of normoxic control. Cultures were exposed to OGD conditions over time. Cell viability was then measured with trypan blue assay. Data are expressed as means \pm SEM (n= 3-5). All treatment wells contained 0.1% DMSO. Analysis with one-way ANOVA showed a significant trend [$F(4,14)= 12.36$, $P= 0.0002$] and Newman-Keuls post-hoc analysis reveals that 6 and 8 hrs of OGD significantly reduced viability from normoxic controls (6 hrs $P<0.001^*$, 8 hrs $P<0.05^{**}$).

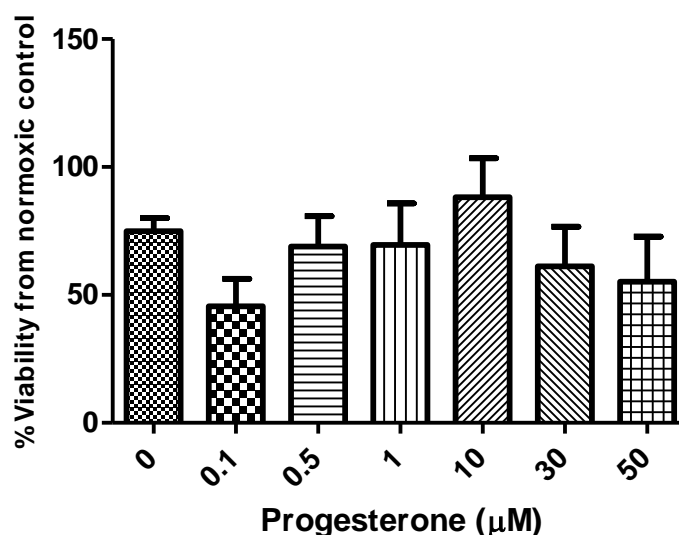
OGD conditions were then applied to cells with increasing progesterone concentrations at 6 and 8 hrs. Progesterone was found not to effect viability compared to OGD controls over these incubation periods (6 hrs [$F(6,37)= 0.87$, $P= 0.7162$], 8hrs [$F(6,30)= 0.74$, $P= 0.6255$]) (figures 3.13-3.14).

Figure 3.13: SHSY5Y viability after 6 hrs OGD, in the presence of different concentrations of progesterone using the trypan blue assay



Cell viability is expressed as % of normoxic control. Cultures were exposed to OGD and cell viability was measured with trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM (n=6-7). Analysis with one-way ANOVA reveals no significant difference in cell viability between treatment groups [$F(6,37)= 0.87$, $P= 0.7162$].

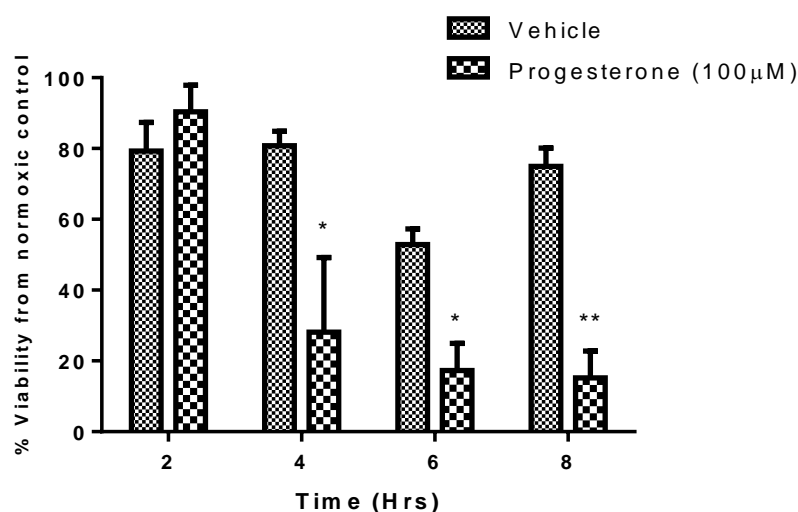
Figure 3.14: SHSY5Y viability after 8 hrs OGD, in the presence of different concentrations of progesterone using the trypan blue assay.



Cell viability is expressed as % of normoxic control. Cultures were exposed to OGD and cell viability was measured with trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM of (n=6-7). Analysis with one-way ANOVA reveals no significant difference in cell viability between treatment groups [$F(6,30)= 0.74$, $P= 0.6255$].

However, progesterone at 100 μ M was found to reduce viability further with increasing OGD incubation from 4 hrs onwards, indicating that high progesterone concentrations at 4 hrs or longer exacerbate rather than reverse OGD-induced neuronal toxicity [$F(1,23)=29.46$, $P<0.001$] (figure 3.15).

Figure 3.15: SHSY5Y viability of vehicle and progesterone, with increasing exposure to OGD using the trypan blue assay.



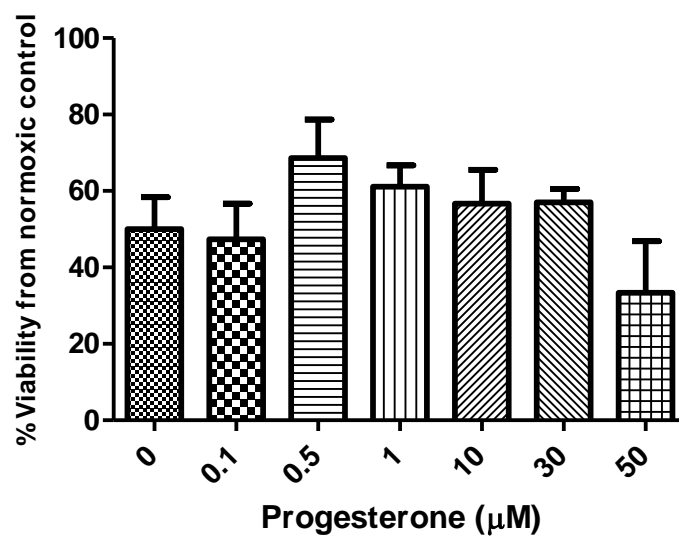
Cell viability is expressed as % of normoxic control. Cultures consisted of control (untreated cells) and progesterone (100 μM)-treated cells, exposed to OGD conditions over time. Viability was then measured with the trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM (n=3-6). Analysis with two-way ANOVA showed a trend for progesterone to significantly reduce viability compared to OGD control [$F(1,23)=29.46$, $P<0.001$]. Bonferroni post-hoc analysis revealed that the trypan blue assay showed significantly reduced viability at 4-6 hrs of OGD (2-6 hrs $P<0.01^*$, 8 hrs $P<0.001$).

3.3.6 The effect of mifepristone on SHSY5Y cells in OGD conditions using the trypan blue viability assay

Mifepristone was added to SHSY5Y cells, which were treated with increasing concentrations of progesterone to determine if cell viability would be affected in OGD conditions. Previously, SHSY5Y cell viability was determined to be significantly reduced by 6 hrs of OGD conditions (figure

3.12). Therefore, this period of OGD was used to observe the affects of mifepristone. The addition of mifepristone with increasing progesterone concentrations in OGD conditions had no effect on cell viability [$F(6,16)=1.53$, $P=0.2304$] (figure 3.16).

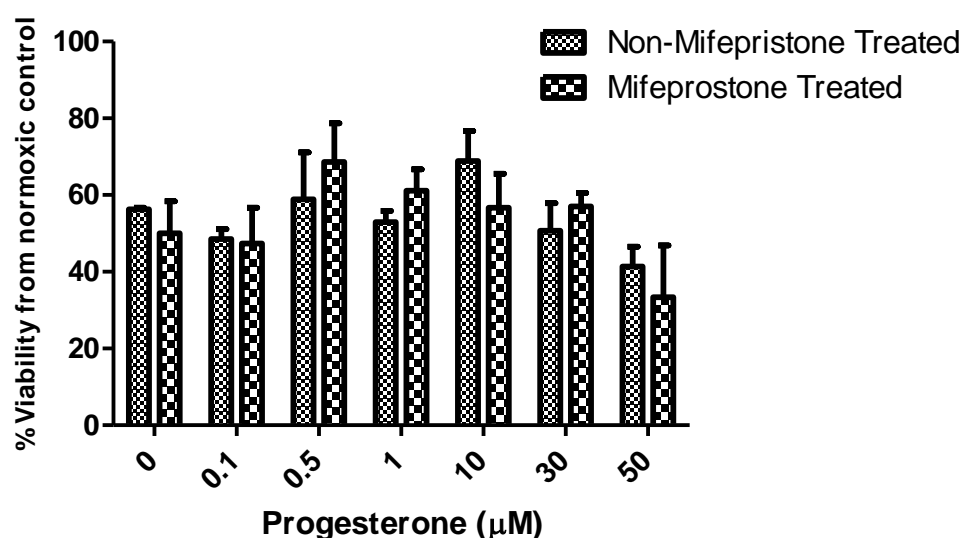
Figure 3.16: SHSY5Y viability in the presence of mifepristone and increasing progesterone concentrations, after 6 hrs exposure to OGD using the trypan blue assay



Cell viability is expressed as % of normoxic control. Cultures were exposed to 6 hrs OGD with mifepristone (10 μM) and increasing concentrations of progesterone (0-50 μM). Viability was then measured with trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM ($n=3-6$). Analysis with one-way ANOVA revealed no significant differences between progesterone concentrations, in the presence of mifepristone in OGD conditions [$F(6,16)=1.53$, $P=0.2304$].

A direct comparison between increasing progesterone concentrations in OGD conditions with and without mifepristone revealed no significant effect of the progesterone receptor antagonist [$F(1,32)= 0.01$, $P= 0.9152$] (figure 3.17).

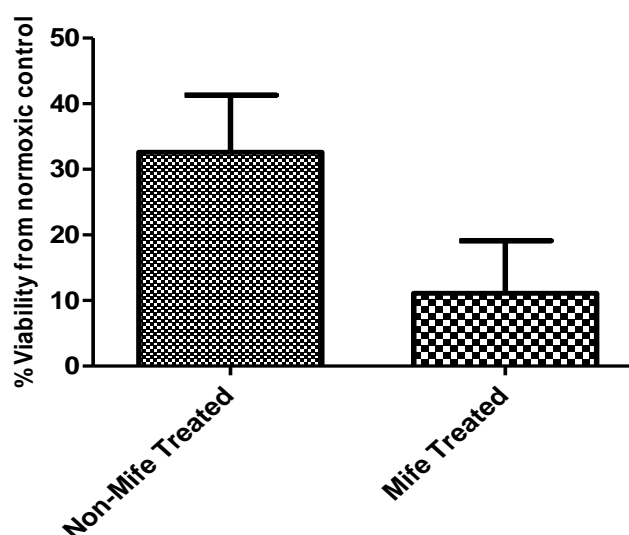
Figure 3.17: SHSY5Y viability with and without the presence of mifepristone and increasing progesterone concentrations, after 6 hrs exposure to OGD using the trypan blue assay



Cell viability is expressed as % of normoxic control. Cultures were exposed to 6 hrs OGD in increasing concentrations of progesterone (0-50 μM) with and without mifepristone (10μM) present. Viability was then measured with trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM ($n= 3-6$). Analysis with two-way ANOVA revealed the presence of mifepristone had no significant effect on viability compared to non-mifepristone treated cells with increasing progesterone concentrations in OGD conditions [$F(1,32)= 0.01$, $P= 0.9152$].

As shown previously, progesterone at 100 μ M was found to further decrease cell viability compared to untreated cells in OGD conditions. A comparison of progesterone's effects in the presence and absence of mifepristone found no significant difference in cell viability ($P=0.1437$) (figure 3.18)

Figure 3.18: No effect of mifepristone on attenuated SHSY5Y viability in the presence of progesterone (100 μ M), after 6 hrs exposure to OGD using the trypan blue assay.



Cell viability is expressed as % of cells in normoxic conditions. Cell cultures were incubated with progesterone (100 μ M), in the presence and absence of mifepristone (Mife 10 μ M) for 6hrs in OGD conditions. Cell viability was then measured using trypan blue assay. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM ($n= 3$). There was no significant effect of mifepristone (T-test) despite a tendency for the antagonist to enhance progesterone-mediated reduction in cell viability ($P=0.1437$).

Table 3.1: Overview of main experimental results

	<u>Cell line</u>	<u>Conditions</u>	<u>Treatments</u>	<u>n</u>	<u>Main Findings</u>
OGD Microglial Cell Experiments	BV-2	4hr normoxic	Vehicle and Prog (30µM)	1 (4 repeats in one experiment)	No decrease in viability from prog (prog higher viability), no difference in NO production
	BV-2	4hr OGD	Prog (0-30µM)	1 (4 repeats in one experiment)	No decrease in viability from prog, no difference in NO production
	BV-2	6hrs OGD, 24 hrs re-oxygenation	Prog (0-30µM)	1 (8 repeats in one experiment)	Viability decreased with increasing concentrations of prog, no difference in NO production
	BV-2	8hrs OGD, 24 hrs re-oxygenation	Normoxic vs. OGD	1 (8 repeats in one experiment)	Cell death too severe after 8hrs OGD followed by 24hrs re-oxygenation
	HAPI	6hrs OGD, 24 hrs re-oxygenation	Prog (0-10µM)	1 (8 repeats in one experiment)	Viability decreased with increasing concentrations of prog, no difference in NO production
Microglial LPS Experiments	BV-2	Normoxic (24 hrs)	LPS, Prog (50µM), Mife, LPS + Mife, Mife + Prog (50µM)	3	Viability unchanged by treatments
	BV-2	Normoxic (24 hrs)	LPS, Prog (50µM), Mife, LPS + Mife, Mife + Prog (50µM)	3	LPS and Mife increased NO production
	BV-2	Normoxic (24 hrs)	LPS + Prog (0-50µM)	3	Viability unchanged between treatments, LPS and Mife increased NO production
	BV-2	Normoxic (24 hrs)	LPS + Mife + Prog (0-50µM)	3	Viability unchanged between treatments, LPS and Mife increased NO production
	BV-2	Normoxic (24 hrs)	LPS + Prog (0-50µM), LPS + Mife + Prog (0-50µM)	3	Mife does not change NO production
OGD Neuronal Cell Experiments	SHSY5Y	Normoxic (2-8hrs)	Vehicle vs. Prog (100µM)	3	Viability unchanged between vehicle and progesterone
	SHSY5Y	OGD (0-8hrs)	Vehicle	3-5	6 and 8hrs OGD decreased viability compared to normoxic control
	SHSY5Y	6hrs OGD	Prog (0-50µM)	6-7	Viability unchanged between progesterone concentrations
	SHSY5Y	8hrs OGD	Prog (0-50µM)	6-7	Viability unchanged between progesterone concentrations
	SHSY5Y	OGD (0-8hrs)	Vehicle vs. Prog (100µM)	3-6	Prog reduced viability compared to vehicle
	SHSY5Y	6hrs OGD	Prog (0-50µM) and Mife	3-6	Viability unchanged between progesterone concentrations
	SHSY5Y	6hrs OGD	Prog (0-50µM) with Mife and without	3-6	Viability unchanged with or without Mife present
	SHSY5Y	6hrs OGD	Prog (100µM) with Mife and without	3	Viability unchanged with or without Mife present

Lipopolysaccharide (LPS, 100ng/ml), Progesterone (Prog), Mifepristone

(Mife, 10µM), Nitric Oxide (NO), Oxygen Glucose Deprivation (OGD)

3.4 DISCUSSION

There is considerable evidence to suggest that microglial activation precedes and predominates over macrophage infiltration into the brain after cerebral ischaemia (Jin et al., 2010). Active microglia, are particularly detrimental for the injured brain and contribute to infarct volume (Schilling et al., 2003; Schilling et al., 2005). The iNOS gene is transcriptionally activated in active microglia, resulting in the production of NO and substantial evidence supports the involvement of NO in ischaemic brain injury (Moro et al., 2004). Experiments in this investigation shows this, as background NO concentrations in HAPI cells, which are activated cells ($9.11 \pm 0.18 \mu\text{M}$) are greater compared to non activated BV-2 cells ($1.54 \pm 0.89 \mu\text{M}$). In the development of ischaemia, NO produced by neuronal nitric oxide synthase (nNOS), followed by inducible iNOS, contributes to the development of brain injury (del Zoppo et al., 2000). Progesterone has been shown to suppress the inflammatory response and iNOS expression following cerebral ischaemia *in vivo* (Gibson et al., 2005b). Also, it has been observed that progesterone decreases iNOS mRNA expression and iNOS promoter activity in macrophages *in vitro* (Lieb et al., 2003). This investigation supports the role of progesterone in attenuating NO production (figure 3.10)

Although, primary cell lines are, perhaps, a more valid model, being directly derived from tissues, the time required for tissue preparation and their relatively short life-span can limit their use. Therefore, immortalised cell lines were used in this investigation. Despite the associated issues with immortalised cell lines of stability, variability, quantity of cells, *in vitro* senescence, along with re-differentiation and subsequent loss of function (Allen et al., 2005), *in vitro* immortalisation enhances tissue survival in culture and cells still retain differentiated traits of the original tissue,

making them more convenient and practical for use than primary cultured cells. Cell lines used in this investigation, have been shown to behave similarly to primary cells in relation to NO production. Hovath *et al* (2008) found the microglia cell lines BV-2 and HAPI cells, to respond similarly to primary microglial cells, in producing NO due to LPS treatment (Horvath *et al.*, 2008), and SHSY5Y cells have been used in other investigations involving OGD conditions (Lorenz *et al.*, 2009; Pei and Cheung, 2003). As a neuronal model, SHSY5Y cells retain many properties of human neuronal cells and can be easily maintained under cell culture conditions (Pei and Cheung, 2003).

The aim of the present chapter was to investigate whether OGD, which is a simple *in vitro* model, of ischaemia, would affect microglial viability and to determine whether progesterone could reduce or exacerbate cell death. Cells under OGD or re-oxygenation conditions were not exposed to serum and were near confluency prior to OGD. Therefore, what was measured was cell death and not proliferation. Microglial function was also investigated by examining whether microglia could be stimulated to produce NO and to observe any effects of progesterone treatment. The mode of action of progesterone was investigated using mifepristone which is a competitive progesterone receptor antagonist, albeit with partial agonist activity. SHSY5Y human neuroblastoma cells were used as a model of cerebral neurons and both Pei *et al* (2003) (Pei and Cheung, 2003) and Lorenz *et al* (2009) (Lorenz *et al.*, 2009) have used SHSY5Y cells to investigate ischaemia.

Different cell viability assays were evaluated, to ascertain if they would be sensitive enough to detect cell death from OGD, and to detect any effects of progesterone treatment. The Neutral Red assay was used to measure cell viability in microglial cells and was found to be sensitive enough to

detect difference in both BV-2 and HAPI cells, in terms of reduced viability in OGD conditions and differences in effects of progesterone treatment. MTT, risazurin and trypan blue viability assays were investigated in SHSY5Y cells. It was found that, MTT was inconsistent in detecting cell death in OGD conditions and with progesterone treatment, possibly due to methodological problems such as the cytotoxicity of MTT formazan which makes it difficult to remove cell culture media from culture wells and MTT formazan needles to form causing cells to float, allowing formazan to be aspirated with culture media, resulting in inconsistent results. The risazurin assay was able to detect differences due to progesterone treatment, but did not indicate significant cell death in OGD or OGD followed by re-oxygenation. The Trypan Blue assay was able to detect significant reductions in cell viability after 6hrs of OGD and was further used in OGD and progesterone experiments involving SHSY5Y cells.

Initial experiments were to test whether OGD conditions would affect microglial viability and whether progesterone would either exacerbate or attenuate cell death. Cells were challenged with deoxygenated, non-glucose-containing SFM and placed in a hypoxic chamber to mimic ischaemic conditions. Production of NO from primary microglia and microglia cell lines (including BV-2) in hypoxic conditions without any pre-activation, has been demonstrated in other studies (Lu et al., 2006; Son et al., 2009), although Park *et al* found that a period of re-oxygenation was required to produce NO after hypoxia (Park et al., 2002). Reperfusion injury *in vivo* is caused when the blood supply returns to tissues after a period of ischaemia, causing inflammation and oxidative damage by the influx of oxygen and glucose. Exposing cells to OGD, followed by re-oxygenation in media with glucose, was an attempt in the current experiments to mimic reperfusion injury *in vitro*. After exposure to OGD

followed by re-oxygenation, viability was decreased in BV-2 cells, and progesterone increased cell death, compared to non-treated cells in OGD conditions. This was unexpected, as *in vivo* studies have found progesterone to be neuroprotective (Gibson et al., 2005b; Gibson and Murphy, 2004). However, Murphy *et al* (2000) did find daily progesterone administration of progesterone for a week prior to ischaemia exacerbated brain injury in overiectomised rats. The authors speculated that the detrimental effects was due to modulation of the GABA system by a sharp decline of progesterone after the pre-stroke treatment (Murphy et al., 2000). This is unlikely to be the case in this investigation, as cells were continually treated through OGD and re-oxygenation. Necrosis from ischaemia is characterised by cellular metabolism failure and loss of membrane integrity (Padosch et al., 2001). Progesterone could possibly aggravate this further as steroid hormones, particular progesterone, can readily insert into bilayers and perturb plasma membrane functions (Snart and Wilson, 1967), resulting in increase cell death.

OGD did not induce NO production in BV-2 cells and so another cell line was investigated. HAPI cells could be considered to model more closely to activated cells and more likely to produce NO. These cells were subjected to the same condition, in order to determine if the lack of NO stimulation was BV-2 specific. HAPI cells were found to be more vulnerable to OGD conditions and had reduced viability when exposed to the same length of OGD and re-oxygenation compared to BV-2. Progesterone treatment was also found to increase cell death when compared to untreated cells in this cell line. However, OGD also failed to stimulate NO production in HAPI cells. The lack of NO production was contrary to previous reports, that microglia do not require pre-activation to produce NO in hypoxic conditions. Park *et al*, regard their study as a 'mild ischaemic insult' due to

the presence of glucose in their cultures. In the present study, the removal of glucose, making the "ischaemia" more severe, did not alter the lack of effect by hypoxia on NO production and so the reasons for the difference with previous studies remain unclear. Microglia may require activation conditions, such as LPS or cytokines including TNF- α and IFN γ in order to produce NO. Conventionally, it has been thought that, microglia cells are activated by signals originating from dying neurons (Gehrmann et al., 1995; Gonzalez-Scarano and Baltuch, 1999), and further experiments are required, in order to determine the effects of pre-activation with cytokines TNF- α and IFN γ , ATP etc. in ischaemic conditions. Also, co-cultures with neurones could clarify the interactions involved between microglia and neurones challenged with ischaemia.

Previous studies have utilised LPS to investigate the inflammatory response of microglia without the use of hypoxia (Horvath et al., 2008). Therefore, LPS was used to stimulate NO production in order to investigate the effects of progesterone. Progesterone reduced NO production after challenging microglia with LPS. Also, blocking the PR receptor did not inhibit the attenuating effect of progesterone on NO production, indicating other mechanisms not involving the PR mediated pathway. In a previous study, Drew *et al* found progesterone to also attenuate LPS-stimulated NO production (Drew and Chavis, 2000), although, unlike the present study, the authors did not investigate the use of a PR receptor antagonist to examine the mechanism of the effect. Oestrogen mediated attenuation of microglial NO production has also been demonstrated. Smith *et al* found oestrogen to also reduce NO production by microglia in the presence of LPS and oestrogen receptor antagonist fulvestrant, was found to inhibit oestrogen's attenuating effect on NO production, indicating a receptor-mediated mechanism (Smith et al., 2011). In the current investigation,

100 ng/ml of LPS was used and no significant loss of viability was found. Smith *et al* used LPS at 200ng/ml, and found it to cause cell death, which was also attenuated by oestrogen. It would be interesting to discover, whether progesterone would also attenuate cell death due to higher levels of LPS. A possible non-PR mediated mechanisms for NO reduction from progesterone is iNOS inhibition through binding to the glucocorticoid response element in murine macrophages (Miller *et al.*, 1996). However, Kohmura *et al* found progesterone mainly uses a receptor different from the glucocorticoid receptor as well as PR (Kohmura *et al.*, 2000), but progesterone could possibly use the glucocorticoid receptor when at high enough concentrations (Werb *et al.*, 1978).

In this investigation, the PR antagonist mifepristone did not alter progesterone's ability to attenuate LPS-stimulated NO production in microglia. The study by Lorenz *et al* supports our findings, as they found that the protection of cortical neurones was not abolished by oestrogen and progesterone nuclear receptor antagonists, indicating a non-classical steroid mechanism involved in neuroprotection (Lorenz *et al.*, 2009). Although, a recent study found PR to be essential for early endogenous neuroprotection in PR knockout mice after transient MCAO, interestingly they also found rapid activation of cerebral biosynthesis of progesterone and 5 α -dihydroprogesterone (Liu *et al.*, 2012). Neuroprotection has been reported involving multiple mechanisms of progesterone-mediated actions, suggesting diverse interactions with different pathophysiological characteristics of stroke injury (Gibson *et al.*, 2009). This current investigation has shown that attenuation of NO production in microglia by progesterone may not involve the classical PR. Although, mifepristone has a higher affinity for PR than progesterone, higher concentrations of the antagonist may be required to block the effects of progesterone.

Progesterone acts through a variety of receptors, and neuroprotection has been shown to be involved in regulating expression of trophic factors, via classical stimulation of PR, including the classical nuclear PRA and PRB types (Gonzalez SL, 2004). PR are not only influenced by progesterone, but also indirectly by oestrogen via oestrogen receptors, which up-regulate the expression of progesterone receptors (Kastner et al., 1990). A reductionist *in vitro* cell line approach, such as the current investigation, has the advantage of not having counter regulations as would be present in more complex systems, potentially enabling mechanisms to be identified more clearly. Other routes of progesterone signalling operate through non-genomic interactions. These actions, in general operate over a rapid time frame that occurs in seconds, to minutes compared to genomic actions, which take hours to days (Gellersen et al., 2009). In the current investigation, NO production was only measured after 24 hrs of exposure and so it can not be confirmed when the attenuating effects of progesterone began. Although, this investigation reveals PR is not involved in the attenuation of NO production by progesterone, indicating rapid non-genomic interactions. Measuring NO production over time and observing when NO levels begin to diverge from progesterone treated and non-treated cells could clarify the time scale of progesterone action. In terms of progesterone action on SH5YSY viability in OGD conditions, 100 μ M of progesterone from 4 hrs onwards exacerbates cell death and is unaltered at 6 hrs in the presence of mifepristone, indicating PR is not involved in progesterone's deleterious actions. Cell viability was unchanged at 2 hrs of OGD with 100 μ M of progesterone present, ruling out signalling from a non-genomic route being responsible in cell death by progesterone. Therefore, another mechanism other than the non-genomic and PR signalling routes is causing cell death. It is likely that high concentrations of progesterone is exacerbating the loss of membrane integrity, as explained

earlier, for increased cell death in microglial cell lines in the presence of progesterone in OGD conditions.

There are also, progesterone receptors unrelated to nuclear receptors, which have been described recently, including a number of membrane-bound receptors. Both progesterone receptor membrane component 1 (PGRMC1) and the related PGRMC2 belong to the membrane-associated receptor family, which are widespread in eukaryotes (Cahill, 2007). Other membrane progesterone receptors, which are unrelated, have been discovered. At least three sub-types of the seven transmembrane progesterone adinoplectin Q receptor have been described (α , β , γ), although their biological significance still needs to be explored (Thomas, 2008). These membrane progesterone receptors display high affinity, limited capacity, displaceable and specific progesterone binding. They are also coupled to inhibitory G proteins that reduce adenylyl cyclase activity (Thijssen, 2009). The transmembrane domain 7TMPR β is another receptor, which is a target for progesterone (Brinton et al., 2008). Also, the progesterone membrane binding site 25-Dx may also be involved in neuroprotection (Labombarda et al., 2003). These non-nuclear receptors are possible targets for progesterone's actions rather than via the classical PR. Therefore, it would be useful to observe the effects of progesterone on cyclic adenosine monophosphate (cAMP) formation in microglia and neurones, and to test for G protein involvement, for example with [³⁵S]GTP γ S binding. Mifepristone's antagonistic action has only been observed towards the classical progesterone and glucocorticoid receptor (Cadepond et al., 1997), but has not been reported to affect these non-nuclear receptors.

Glutamate overload is a key neurotoxic effect that is thought to occur, following brain injury, including acute ischaemic stroke. The molecular

mechanisms of glutamate-induced excitotoxicity have been investigated with *in vitro* neuronal models, and progesterone has been shown to protect against excitotoxicity in dissociated cell cultures (Luoma et al., 2011; Mannella et al., 2009; Nilsen and Brinton, 2003; Ogata et al., 1993). Ischaemia models have been used to reduce viability in neuronal cultures and progesterone was shown to provide protection in a concentration-dependent manner via GABA_A receptor activation, thereby reducing excitotoxicity in purkinje neurons (Ardeshiri et al., 2006). In this present investigation, progesterone was found not to protect microglia and neuronal cell lines in terms of cell viability. In relation to GABA system modulation in microglia by progesterone, stressed neurons from OGD may need to be present to release glutamate and activate microglia (Kaushal and Schlichter, 2008). However, pure microglia cultures have been shown to release glutamate after stimulation with LPS (Barger et al., 2007), although LPS stimulation may not necessarily represent the inflammatory response in stroke. The interaction of co-neuronal and microglial cultures would better represent the effects of progesterone on GABA system modulation *in vivo*.

A possibility for why neuroprotection was not observed, in the presence of progesterone in the present study may be due to the cell types employed. Coughlan *et al* found progesterone to protect PC12 neurons deprived of trophic support but not cerebellar granule neurons (Coughlan et al., 2009). It is possible that cells from different brain regions or cell lines may require a combination of sex steroids present in order to benefit from neuroprotection. Some neurones have been observed to respond in a sexually dimorphic manner, while other neurones in different brain regions do not. Other studies found cellular stress responses, leading to cell death to be gender-dependent, (Du et al., 2004; Lieb et al., 1995), while others

found no such sex differences in other cell line responses to ischaemic insult (Ardeshiri et al., 2006). Cellular responses to ischaemia can vary due to gender and so responses to therapy could be affected in a similar manner. In previous studies evaluating neuroprotectants, differences in outcomes have been observed due to gender (Liu et al., 2009b). In the present investigation, SHSY5Y neuroblastoma cells which are genetically female were used (Biedler et al., 1973). Although, progesterone neuroprotection was not found in this cell line, a male neuronal cell line may respond differently, such as SMS-KCN (Reynolds et al., 1986). Interestingly, in a systematic review of progesterone in the treatment of experimental brain injury, progesterone was found to be more beneficial in males compared to females (Gibson et al., 2008b). Lorenz *et al* found combined oestrogen and progesterone, but not the hormones individually, prevented neuronal cell injury in cortical neurons. The same study also found midbrain neurons and SHSY5Y neuroblastoma cells, as used in the present study were not protected, indicating that neuroprotection can be cell or region-specific (Lorenz et al., 2009). Although, they found SHSY5Y cells not to be protected by progesterone, their cell injury model involved glucose-serum deprivation and did not include hypoxia. Oxygen deprivation, as employed in the present investigation is, therefore, a more relevant model for ischaemic stroke. Ischaemic cell damage is more severe and proceeds faster compared to either glucose or hypoxic injury alone. The decline of ATP depends not only on hypoxia and attendant cessation of oxidative phosphorylation, but also on failure of anaerobic glycolysis as found in ischaemic conditions. These mechanisms present extra possible targets for progesterone, which would not be present with glucose-serum deprivation alone. Also, reperfusion damage can be observed, when the availability of oxygen and nutrients during reperfusion allows ischaemic cells to restore ATP pools which can cause additional cell damage, due to

the energy requirements of apoptosis (Saikumar et al., 1998). An example of a possible target for progesterone neuroprotection, is that oxygen free-radicals have been implicated in the pathogenesis of reperfusion injury (McCord, 1985).

Long incubation periods (6-8 hrs) with high concentrations of progesterone at 100 μ M exacerbated SHSY5Y, neuroblastoma cell death in ischaemic conditions. Brain progesterone concentrations are thought to closely mirror circulating serum levels, and the concentrations of progesterone that exacerbated ischaemic cell death in the present study could be considered to be pharmacological and above those found even during human pregnancy, which is approximately 12.72–63.6 nM (Erickson, 1995). It is unlikely, if progesterone were to go into human stroke trials, concentrations of progesterone at the higher end of the dosage range investigated here could be achieved for prolonged periods *in vivo*, due to metabolism, clearance and bioavailability. It is more likely if an infusion method were deployed in a clinical trial for progesterone in stroke, progesterone concentrations would reach similar levels to those achieved by the clinical trials evaluating progesterone as a neuroprotectant in traumatic brain injury (TBI) (Wright et al., 2007; Xiao et al., 2008). In a preliminary study prior to these trials, steady-state serum concentrations of progesterone reached 337 ± 135 ng/ml, following continuous intravenous infusion in patients with acute moderate to severe TBI injury (Wright et al., 2005). This method of infusion was utilised in the progesterone TBI trials described previously, which found progesterone to be beneficial and safe. This concentration of progesterone is approximately 1 μ M at which no reduction in viability of SHSY5Y cells during ischaemia was observed during the present investigation. Ischaemia followed by re-oxygenation over a long period of time with progesterone 100 μ M reduced

SHSY5Y viability in the present study, revealing toxicity of progesterone in these conditions. However, as mentioned previously these concentrations of progesterone are unlikely to be encountered *in vivo* for extended periods, but this emphasizes why pharmacokinetics and dose response studies are important.

The pharmacokinetics of progesterone after 8 mg/kg via i.p. injection in mice, found plasma progesterone concentrations to be 110.28 ng/ml (0.35 μ M). Also, the *in vitro* effect of progesterone, inhibiting NO production has a half maximal effective concentration of approximately 5 μ M. These concentrations are lower in comparison to the steady-state serum concentrations achieved in the preliminary TBI clinical study (337 ± 135 ng/ml or 1.07 μ M) (Wright et al., 2005). However, care must be taken when directly comparing progesterone concentrations between species and dosing regimens because of different anatomy, biochemistry and physiology. The dose of 8 mg/kg of progesterone via i.p. injection, has been previously found to be neuroprotective in mice (Gibson et al., 2005b; Gibson et al., 2011; Gibson and Murphy, 2004; Liu et al., 2012). In relation to the concentrations used to inhibit NO production *in vitro*, BV-2 microglia cells are mouse derived. Therefore, the concentration range should be relevant to the pharmacokinetic concentrations found in mice and where the cells reside i.e. the brain. Brain progesterone concentrations after 8 mg/kg after i.p. injection reached a peak of 268.27 ng/g (0.85 μ M) and mini-pump delivered approximately 70 ng/g (0.22 μ M) when at steady state over a period of 48 hrs. The use of lower progesterone concentrations, and over a longer treatment time, may be a better comparison to the expected concentrations found *in vivo*.

In summary, progesterone reduced NO production, after challenging microglia with LPS and that the nuclear progesterone receptor is not

involved. Neither of the microglial cell lines, BV-2 and HAPI cells produced elevations in NO formation in ischaemic conditions. The *in vitro* OGD model of ischaemia, reduced viability of both microglial and neuronal cells. Also, high pharmacological concentrations of progesterone exacerbated ischaemic injury. OGD *in vitro* modelling is relatively simple to perform and has the advantage of allowing key mechanisms to be identified and studied, without counter regulations present, that are found in complex systems. Progesterone's ability to reduce NO has been demonstrated *in vitro* previously in microglia. However, this present investigation has identified, that PR does not have a major role in progesterone's attenuation of NO production in a microglia cell line. Although, the involvement of PR in the mechanism of neuroprotection by progesterone has also been identified in a *in vivo* study of PR knock-out mice (Liu et al., 2012). However, further investigation is required on primary cells and *in vivo* to determine the importance of progesterone, PR and NO formation by microglia in stroke.

CHAPTER 4

Progesterone treatment and focal ischaemia on mouse models

Publications contributing to this chapter:

Sustained release of progesterone, enhances functional recovery following transient cerebral ischaemia in male mice (Submitted to journal)

4.1 INTRODUCTION

Progesterone delivery via osmotic mini-pump (in conjunction with bolus i.p. loading dose) was found to be an effective mechanism for delivering progesterone (Wong et al., 2012). We went on to determine whether progesterone delivery via osmotic mini-pumps in an experimental stroke model, induced via middle cerebral artery occlusion (MCAO), is valid. Sustained release of progesterone via mini-pumps could offer a more suitable dosing method with the advantages of reducing peaks and troughs in drug levels, the stress associated with repeated injections, and diminishing levels of release over time as seen with pellet implants. Infusion methods are commonly used clinically, to maintain drug concentrations, and osmotic mini-pump release of agents mimics this approach. Also, tapered progesterone withdrawal has been shown to promote long-term recovery after TBI (Cutler et al., 2006b). Stroke involves similar pathological mechanism as TBI. Therefore, it is possible that tapered progesterone withdrawal could promote long-term recovery in stroke.

Numerous neuroprotective drugs that appear to be effective in animal models have failed when reaching clinical trials (O'Collins et al., 2006). A possibility may be due to the failure to consider the complexities of human disease. The majority of studies, involving animals to test the efficacy of potential neuroprotectants, such as progesterone, have involved young, healthy and male animals, which do not represent the demographic of stroke patients. Co-morbidities are common in patients with stroke and have been shown to be negatively correlated with functional outcome (Karatepe et al., 2008). Although, the impact of these co-morbidities such as age, diabetes and hypertension, have not been fully investigated, they are known to have a major influence in terms of stroke outcome. The

factor of age in combination with other co-morbidities, such as cardiac co-morbidities, congestive heart failure, hypertension, hyperlipidemia and diabetes, results in a poorer stroke outcome compared to individual co-morbidities on their own (Turhan et al., 2009).

Age is the single most important risk factor for stroke and the incidence of stroke increases with age (Feigin et al., 2003; Herman et al., 1982; Niessen et al., 1993). For each successive 10 years after the age of 55, the stroke rate more than doubles in both men and women (Brown et al., 1996; Wolf et al., 1992). Stroke incidence rates are 1.25 times greater in men, but because women tend to live longer than men, more women than men die of stroke each year (Sacco et al., 1997). Stroke severity at onset and patient age are the most important factors for predicting prognosis (Ingall, 2004). In an epidemiological study involving patients at 80 years old and over, were found to have a higher proportion of conscious impairment at admission, a longer acute ward stay, a higher incidence of total anterior circulation infarct and a lower frequency of lacunar infarct compared to patients under 80 years of age (Lee et al., 2007).

The prevalence and incidence of atherosclerosis increases with age and is a major cause of strokes (Costopoulos et al., 2008). In the ageing process various changes happen in the brain, which can affect the outcome of stroke. Ageing effects neurotransmitter levels, such as dopamine, which decline by around 10% per decade from early adulthood (Mukherjee et al., 2002). Serotonin and brain derived neurotrophic factor levels also fall with increasing age and may be implicated in the regulation of synaptic plasticity and neurogenesis in the adult brain (Mattson et al., 2004). A substance related to neurotransmitter levels, monoamine oxidase, increases with age and may liberate free radicals from reactions that exceed the inherent antioxidant reserves (Volchegorskii et al., 2004).

Other factors that have been implicated in the ageing brain include calcium dysregulation (Toescu et al., 2004), mitochondrial dysfunction, and the production of reactive oxygen species (Melov, 2004). The ageing brain may also suffer from impaired glucose metabolism or a reduced input of glucose or oxygen as cerebrovascular efficiency falls, although reduction in glucose may partly be attributable to atrophy rather than any change in glucose metabolism (Ibáñez et al., 2004).

Along with age, other risk factors have a significant impact on outcome following stroke, including high blood pressure. The global prevalence of high blood pressure is 26.4% of the adult population with increasing incidence (Kearney et al., 2004). A well recognised risk factor for stroke is hypertension and pre-existing hypertension may be present in more than half of stroke patients (Arboix et al., 2004; Goldstein et al., 2011). Hypertension affects both small and large vessels supplying the brain (Rodgers et al., 1996), and there is a strong association between hypertension and mortality following stroke (Andersen et al., 2011; Heuschmann et al., 2004).

At the onset of hypertension, there is a long delay from the onset of high blood pressure to the complications of atherosclerosis related to the phenomenon. In this time frame, a series of changes take place in the cardiovascular system, including the cerebral circulation. These changes, such as vascular remodelling, inflammation, oxidative stress and baroreflex dysfunction etc, may contribute to the pathogenesis of hypertension (Yu et al., 2011). Arterial baroreflex is an important physiological mechanism controlling blood pressure regulation (Persson, 2005). Baroreflex sensitivity, a marker for baroreflex function plays an important role in the pathogenesis and prognosis of atherosclerosis, acronitine-induced arrhythmia and LPS-induced shock in animals (Cai et al., 2005; Shen et

al., 2004; Shu et al., 2004). Hypertension can reduce baroreflex sensitivity by changing vascular dispensability and altered activity in the brainstem portion of the reflex (Bristow et al., 1969). A reduction in baroreflex sensitivity results in vascular changes, arterial stiffness and contributing to a vicious cycle of hypertension complications. Impairment of the Baroreflex has shown to be present in acute ischaemic and haemorrhagic stroke (Eveson et al., 2005; Sykora et al., 2008). In addition, oxidative stress in cerebral blood vessels is induced by hypertension and participates in the structural and functional alterations of cerebral blood vessels, by increasing ROS production in cerebral vessels by angiotensin (Ang) II, via activation of nicotine adenine dinucleotide phosphate (NADPH) oxidase in the vasculature (Sindhu et al., 2005). Compelling evidence suggests oxidative stress plays a critical role in the pathogenesis of hypertension and stroke as a long-term complication (Vaziri, 2004; Vaziri and Rodriguez-Iturbe, 2006).

Hypertension has profound effects on the structure of cerebral blood vessels including mechanical, neural and humoral factors, which all contribute to the changes in the composition and structure of the cerebral vascular wall. Examples include; (i) development of atherosclerotic plaques in cerebral arteries and promotion of arterioles, leading to arterial occlusions and ischaemic injury (Dahlof, 2007; Gkaliagkousi et al., 2009; Lammie, 2002), (ii) lipohyalinosis of penetrating arteries and arterioles supplying the white matter, resulting in small white matter infarcts or brain haemorrhage (Lammie, 2002), (iii) hypertrophy and remodelling of smooth muscle cells in the systemic and cerebral arteries, both of which are aimed at reducing stress on the vessel wall and protecting downstream micro vessels (Baumbach and Heistad, 1988; Laurent et al., 2005), (iv) smooth muscle cells when under high chronic intraluminal pressure undergo

hypertrophy, hyperplasia or rearrangement and therefore grow inward, encroaching into the luminal space of the artery resulting in the narrowing of the artery and increasing wall thickness, (v) vascular stiffening is present in hypertension, increasing pulse pressure (a good predictor of stroke) (Baumbach and Heistad, 1988; Benjo et al., 2007) and, (vi) cerebral blood flow is also altered by hypertension, resulting in intracranial arterial vasculature dilation, leading to a weakened ability for additional vasodilatation in response to ischaemic events and a higher risk of subsequent accidents (Yu et al., 2011).

Inflammation is a vital process that leads to changes in vascular wall integrity and is a common pathological mechanisms of damage in a variety of vascular diseases, including atherosclerosis and cerebral aneurysms (Chyatte et al., 1999; Henry and Chen, 1993). Studies have shown that the biomarkers of inflammation can predict the risk of primary ischaemic stroke (Tzoulaki et al., 2007). Inflammatory markers such as C-reactive proteins, IL-6, leukocyte elastase, lipoprotein (a), intercellular adhesion molecule (ICAM)-1 and E-selectin are consistently higher in people prone to develop stroke in comparison to those not at elevated risk. Exacerbated inflammation may also lead to worse outcome following stroke, resulting from an increase of C-reactive protein (CRP) in response to IL-6 (McColl et al., 2009; Wang et al., 2009). Inflammation has been identified as a risk factor for stroke (Mehta et al., 2010; Ridker, 2010).

There are also links between hypertension and changes in the brain. Moderate to high 24 hour ambulatory blood pressure has been related to increased brain atrophy as has increased variability of systolic blood pressure (Goldstein et al., 2002). In Japanese subjects, raised systolic blood pressure was related to grey matter volume loss in a cross sectional study (Taki et al., 2004), and in the Framingham offspring cohort study, a

10 year increase in the risk of having a first stroke was associated with a reduction in cognitive function. The authors suggest that this may be attributable to cerebrovascular related injuries, accelerated atrophy, white matter abnormalities, or asymptomatic infarcts (Elias et al., 2004). The relation of cerebral vasculature to cognitive function is not surprising because the ability of the microvasculature to respond to metabolic demand falls with increasing age, and moreover, functional adult neurogenesis (Finch, 2003; Kempermann et al., 2004).

Diabetes Mellitus is also a recognised risk factor for stroke (Tuomilehto et al., 1996), although the benefit of glycaemic control on primary and secondary prevention for stroke and other micro-vascular events was not established until relatively recently (Wilcox et al., 2007). Sub-group analysis from the 'Prospective pioglitazone clinical trial in microvascular events' (PROactive) 04 study found for the first time the positive effect of glucose-lowering therapy (pioglitazone) in secondary stroke prevention (Wilcox et al., 2007). The population risk of stroke attributable to diabetes was found to be 18% in men and 22% in women (proportion of cases which potentially could be prevented by eliminating diabetes) (Stegmayr and Asplund, 1995). Diabetic patients have higher hospital and long-term stroke mortality, greater pronounced residual neurological deficits, more severe disability and prolonged hospital stay after acute cerebrovascular occurrence (Mankovsky and Ziegler, 2004). Patients with diabetes have higher mortality after stroke onset compared to non-diabetic patients in the first three months (Oppenheimer and Hoffbrand, 1985). This trend in mortality is also present one year after onset in diabetic patients versus non-diabetics (50% vs 25%) (Oliveira et al., 1988). Longer term studies found mortality rates to be 1.7 times at one year, and 2 times higher at 5 years after the onset of stroke despite similar fatality rates at 28 days

(Sprafka et al., 1994). Only 20% of people with diabetes survive over 5 years after first incidence of stroke and half die within the first year (Asplund et al., 1980; Olsson et al., 1990).

There is convincing evidence to suggest that diabetes mellitus is a strong independent risk factor of stroke and it is associated with increased incidence of recurrent cerebrovascular events. Diabetes is a risk factor mainly for ischaemic stroke, while its association with haemorrhagic stroke remains controversial. Hyperglycaemia is common in stroke patients. However, it is not known whether hyperglycaemia independently influences the course and outcome of stroke (Mankovsky and Ziegler, 2004).

The reasons for worse outcome of stroke in diabetics are not fully understood. A possibility is the higher prevalence of atrial fibrillation, arterial hypertension, heart failure and prior myocardial infarctions in diabetic stroke patients compared to non-diabetic patients (Alter et al., 1993; Carlberg et al., 1991; Olsson et al., 1990; Stegmayr and Asplund, 1995). Chronic hyperglycemia, duration, type and complications in diabetes, insulin resistance and its associated phenomena may significantly contribute to an elevated stroke risk by amplification of the harmful effect of existing risk factors or acting independently (Mankovsky and Ziegler, 2004). Aggressive control of arterial hypertension and dyslipidemia decreases the risk of stroke substantially, while the importance of glucose control for stroke prevention remains unproven (Mankovsky and Ziegler, 2004). Recent data supports the suggestion that reduced cerebral blood flow and blood brain barrier damage may be behind the aggravating effects of hyperglycaemia and diabetes (Kamada et al., 2007; Kawai et al., 1998). The exacerbation of inflammatory processes may also contribute to worse outcome (Ding et al., 2005; Martin et al., 2006; Panes et al., 1996).

Diabetes can also directly affect the brain to worsen the outcome of stroke. Type-2 diabetes mellitus is associated with atrophy, white matter hyperintensities, lacunar infarcts and vascular lesions of the brain (Tiehuis et al., 2008; van Harten et al., 2006). Successful stroke recovery hinges on the ability of the brain remap sensory and motor functions to surviving, functionally homologous brain regions. Diabetes have been shown to impair cortical plasticity and functional recovery following ischaemic stroke in type-1 diabetic mice (Sweetnam et al., 2012).

Further investigation is required to explore the neuroprotective effectiveness of progesterone in these co-morbid animals, particularly the major risk factors in stroke of age, diabetes and hypertension. Stroke outcomes found in co-morbid animal models can differ compared to healthy adults (table 4.1).

Table 4.1: Outcomes found in co-morbid models of stroke in comparison to healthy adult animals

<u>Co-morbid Model</u>	<u>Infarct Volume</u>	<u>Oedema</u>	<u>Functional Deficits</u>
Aged	Older animals develop larger infarcts (Alkayed et al., 1998; Hurn and Macrae, 2000; Li et al., 1996)	Reduced oedema formation (Liu et al., 2009a)	Functional recovery suppressed (Popa-Wagner et al., 2007; Tan et al., 2009)
Hypertensive	hypertensive animals have larger infarcts (Duverger and Mackenzie, 1988)	Increased oedema formation (Fenske et al., 1978)	Variable learning deficits and paresis (Okamoto et al., 1975)
Diabetic	Larger infarcts in both transient and permanent models (Duverger and Mackenzie, 1988; Liu et al., 2007; Morikawa et al., 1999)	Increased oedema formation (Morikawa et al., 1999)	Greater functional deficit (Ning et al., 2012)

The aims of the investigation reported here, was to evaluate if progesterone would be effective in conferring neuroprotection, after the onset of experimental stroke, when administered via osmotic mini-pump in adult C57 Bl/6 mice. Also, to evaluate whether the effects of induced experimental stroke on co-morbid models and the neuroprotective effectiveness of progesterone in these models.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 (Project License 40/3207) and were approved by the University of Nottingham ethical review process. Mice were assigned a code by a non-experimenter and assigned randomly to different treatment groups. During all surgical procedures and behavioural tests/analyses the experimenter was blinded to treatment. In order to the appropriate risk factors for stroke, we aimed to compare outcomes following progesterone treatment, in adult, aged, diabetic and hypertensive mice (Table 4.2 for type and mice number). All mice were assigned a code by a non-experimenter and assigned randomly to different treatment groups. During all surgical procedures and behavioural tests the experimenter was blinded to treatment. Group sizes were determined by power analysis (G*Power 3.1.7) based on foot-fault results from Gibson *et al* 2004 (Progesterone 66.77 ± 5.35 %, Vehicle 77.44 ± 5.55 %) and n=8 per group was determined.

Table 4.2: Type and overall number of mice in each experimental group that underwent surgery

Mouse Type	Number of animals per MCAO group (minutes of occlusion)					
	Sham	5	10	15	30	60
Adult C57 Bl/6	5	0	0	0	20	4
Aged C57 Bl/6	0	0	0	4	15	0
Diabetic NOD/ShiLtJ	0	2	0	0	2	0
Hypertensive BPH/2	0	2	2	9	0	0

4.2.1.1 Adult C57 Bl/6

A total of 25 male C57 Bl/6 mice (15-25 weeks) were used, weighing between 25.9 and 41.2g at the time of surgery, 20 of these were subjected to 30 minutes of focal ischaemia and 5 were shams. A small number of 4 male C57 Bl/6 mice (15 weeks) weighing 27.3 and 30g at the time of injection were subjected to 60 minutes of occlusion.

4.2.1.2 Aged C57 Bl/6

In total, 15 aged male C57 Bl/6 mice (61-65 weeks) were used, weighing between 34.10 and 46.5g at the time of injection and undergone 30 minutes of occlusion. There were 4 animals aged 67 weeks, weighing 42.5 and 50.7g at the time of injection were subjected to only 15 minutes of occlusion.

4.2.1.3 NOD/ShiLtJ

NOD/ShiLtJ (Non-obese diabetic) mice are a polygenic strain for type 1 diabetes are characterised as having insulinitis, leukocytic infiltration of the pancreatic islets. They have a marked decrease in pancreatic insulin

content, which occurs in females about 12 weeks of age and several weeks later in males. The onset of diabetes is marked by moderate glycosuria and by non-fasting plasma glucose, higher than 250 mg/dl. Diabetic mice are hypoinsulinemic and hyperglucagonemic, indicating a selective destruction of pancreatic islet beta cells (Makino et al., 1980). Susceptibility to insulin-dependent diabetes mellitus in NOD/ShiLtJ mice is polygenic and affected by environment (including housing conditions), health status, and also diet exert a strong effect on penetrance. The major component of diabetes susceptibility in NOD mice is the unique MHC haplotype ($H2^{g7} = K^d, Aa^d, Ab^{g7}, E^{null}, D^b$). NOD/ShiLtJ exhibit multiple aberrant immunophenotypes including defective antigen presenting cell immunoregulatory functions, defects in the regulation of the T-lymphocyte repertoire, defective natural killer (NK) cells cell function, defective cytokine production from macrophages, impaired wound healing and lack hemolytic complement, C5. These mice also have severely impaired hearing (Serreze et al., 1997).

In total of 4 male NOD/ShiLtJ mice (21 weeks) were used, weighing between 29.8 and 32g at the time of injection for 30 minutes of occlusion. The remaining 11 animals (22 weeks of age) were used to monitor symptoms and to gather background behaviour data, weighing 29.3 and 32.9g at the start of monitoring.

4.2.1.4 BPH/2 hypertensive mice

Hypertensive BPH/2 mice have elevated systolic blood pressures at five weeks of age and by 150 days differ from the BPL/1 (low blood pressure) by 60 mmHg. The BPH/2 strain has a higher heart rate, larger hearts and kidneys, and higher hematocrits than the BPL/1 strain. They also have lower renin, aldosterone and angiotensin I levels compared to the BPL/1

and BPN/3 (normal blood pressure). The original HBP (high blood pressure) and LBP (low blood pressure) selected lines show a number of biochemical and physiological differences which have not been re-examined in the inbred strains. These include differences in brain catecholamines, calmodulin concentrations, heat sensitivity, alcohol preference, and longevity. Hypotensive selected lines live two to three hundred days longer, on average, than the hypertensive selected lines. Biometrical genetic analysis suggested that three to five genes are responsible for the difference in blood pressure between the BPH/2 and BPL/1. A genome scan of an F2 from a BPH/2 x BPL/1 cross found three chromosome locations that cosegregated with blood pressure. Two of these sites were verified by candidate gene cosegregation: angiotensinogen on chromosome 8 and mouse kallikrein binding protein on chromosome 12. The BPH/2 strain is considered a model of chronic hypotension that is rennin independent. This hypertensive strain is also associated with hypertrophy in cerebral arterioles, but not remodelling (Baumbach et al., 2003).

A total of 13 male BPH2/2 mice (28-37 weeks) were used, weighing between 21.9 and 34.8g at the time of occlusion, 8 of these were subjected to occlusion ranging from 5 to 15 minutes and were not treated, while 5 animals received progesterone.

4.2.2 Focal cerebral ischaemia

Anaesthesia was induced by inhalation of 4% isoflurane (in 100% Oxygen) and maintained by inhalation of 1.5% isoflurane. Body temperature was monitored throughout surgery (via rectal probe) and maintained at 37°C ± 0.5°C using a heating blanket (Harvard Apparatus, Edenbridge, Kent, UK). Laser Doppler flowmetry (Moor Instruments, Sussex, UK) was used to

monitor cerebral blood flow. Focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAO) as previously described (Gibson et al., 2005b). Briefly, a small incision was made in the skin overlying the temporalis muscle and a 0.7-mm flexible laser-Doppler probe (model P10) was positioned on the superior part of the temporal bone (6mm lateral and 2mm posterior from bregma), secured by superglue (Loctite). A midline incision was made on the ventral surface of the neck and the right common carotid arteries were isolated and ligated. The internal carotid artery was temporarily occluded using a microvascular clip (Ohwa Tsusho Co., Tokyo, Japan). A nylon filament (Drennan, Stridgewarer, UK), exposed to heat to give a diameter of 180 μ m, was introduced into the internal carotid artery via an incision in the common carotid artery. The filament was advanced approximately 10 mm distal to the carotid bifurcation, beyond the origin of the middle cerebral artery. Relative cerebral blood flow was monitored for the first 5 minutes following MCAO before mice were allowed to return to their home cage. After 5 to 30 minutes of MCAO depending on strain and treatment group, mice were re-anesthetised and the occluding filament was withdrawn back into the common carotid artery to allow reperfusion to take place. At this stage, drugs were then administered and osmotic minipumps were implanted (see drug treatment below). Relative cerebral blood flow was monitored for an additional 5 minutes before the wound was sutured and mice were allowed to recover from the anaesthesia. The relative cerebral blood flow had to rise to at least 50% of pre-ischaemic values for mice to be included in the study and subject to further analyses. Sham-operated mice underwent the same surgical procedure, except that the filament was not advanced far enough to occlude the middle cerebral artery and they received empty of DMSO mini-pumps.

4.2.3 Drug treatment

Following random allocation, C57 Bl/6 (adult and aged) and NOD/ShiLtJ (allocated for MCAO) mice were injected intraperitoneally, at the onset of reperfusion, with a 'loading dose' of either progesterone (USP grade, 8mg/kg in 100% DMSO, Sigma, St Louise, MO, U.S.A) or vehicle (100% DMSO). Mini-pumps, with an infusion rate of 1.0 µl/hr and a reservoir with up to 3 days delivery capacity (Alzet 1003D), were loaded with progesterone solution (50 mg/ml progesterone dissolved at 37°C in 100% DMSO only) or 100% DMSO (placebo). The pumps were submerged in 0.9% sterile saline solution at 37°C over night in order to prime them so that progesterone was delivered immediately after implantation. Mini-pumps were implanted subcutaneously in the back immediately following the onset of reperfusion and administration of the initial loading dose (progesterone or vehicle) as stated above. Hypertensive BPH/2 mice only received the intraperitoneal injection, at the onset of reperfusion, with the 'loading dose' of either progesterone (USP grade, 8mg/kg in 100% DMSO, Sigma, St Louise, MO, U.S.A) or were non-treated.

4.2.4 Assessment of general well-being

After surgery, mice were weighed every day for 7 days as an indication of their general well-being (Gibson and Murphy, 2004). Body weights are presented as a change compared with weight on the day of surgery. Survival rates of animal groups are also presented as % compared to the number of animals undergoing surgery.

4.2.5 Neurological deficit score

Neurological deficit scoring can be used to quantify the different aspects of neurological deficits, such as sensory-motor asymmetry caused by focal cerebral ischaemia. Animals were scored neurologically for focal deficits with the use of a 28-point neurological scoring system based from Clark *et al* (Clark et al., 1998) (table 4.3). We also used a modified version of this whereby animals, that died prematurely, were assigned a score of 29 points i.e. beyond the maximum neurological deficit possible. This is done in a similar fashion as the modified rankin scale to encompass death in analysis.

Table 4.3: Neurological score (28 points)

	0	1	2	3	4
(1) Body symmetry (open bench top)	Normal	Slight asymmetry	Moderate asymmetry	Prominent asymmetry	Extreme asymmetry
(2) Gait (open bench top)	Normal	Stiff, inflexible	Limping	Trembling, drifting, falling	Does not walk
(3) Climbing (gripping surface, 45° angle)	Normal	Climbs with strain, limb weakness present	Holds onto slope, does not slip or climb	Slides down slope, unsuccessful effort to prevent slope	Slides Immediately, no effort to prevent fall
(4) Circling behavior (open bench top)	Not Present	Predominantly one-sided turns	Circles to one side (not constantly)	Circles constantly to one side	Pivoting, swaying or no movement
(5) Front limb symmetry (mouse suspended by its tail)	Normal	Light asymmetry	Marked asymmetry	Prominent asymmetry	Slight asymmetry, no body/limb movement
(6) Compulsory circling (front limbs on bench, rear suspended by tail)	Not Present	Tendency to turn to one side	Circles to one side	Pivots to one side sluggishly	Does not advance
(7) Whisker response (light touch from behind)	Symmetrical Response	Light asymmetry	Prominent asymmetry	Absent response ipsilaterally, diminished contralaterally	Absent proprioceptive response bilaterally

Table shows 28-point neurologically focal deficits scores of 7 categories ranging from 0 for no deficit to 4 for extreme deficit.

4.2.6 Foot fault

The foot-fault task assesses motor impairments of limb function and placing deficits during locomotion in rodents due to damage to brain regions, such as the striatum or motor areas of the cerebral cortex (Rogers et al., 1997). As previously described (Gibson and Murphy, 2004) at 24 hr, 48 hr and 7 days after MCAO or sham surgery, mice were placed on an elevated grid surface (30 x 35 x 31 cm) with grid openings of 2.5 cm². During locomotion on the grid, the number of foot faults made by the ipsilateral and contralateral limbs was counted. Tests consisted of 3 trials of 1 minute each with an interval of 1 minute between trials. The foot faults are expressed as the number of errors made by the contralateral side limbs in % of the total errors made. Modified foot fault is essentially the same as normal foot fault. However, animals which have died or killed for welfare are included in analysis and are scored as 100% contralateral foot slips.

4.2.7 T-maze

MCAO can produce secondary, remote impairment of the hippocampus, which participates in spatial learning and exploratory behaviour. Mice normally alternate at levels significantly above chance, indicating their willingness to explore novel environmental stimuli (Lalonde, 2002). Studies examining neurological deficits in rodents have reported a lower alternation rate in animals with focal ischaemia (Gerlai et al., 2000; Ishibashi et al., 2003b; Itoh et al., 1993; Kadam et al., 2009; Matchett et al., 2007). Also, animals suffering ischaemic brain injury have a tendency to favour the arm ipsilateral to the damaged side (Carlioni et al., 2008) and take longer to complete t-maze trials (Ishibashi et al., 2003a). The T-maze

consists of 3 arms made of grey Perspex, each 41.5 cm long and 6 cm wide, surrounded by walls of transparent Perspex (15 cm high). The start box (6cm x 7.5 cm) is located at the bottom of the central arm. The start box and the entrance of each arm can be closed by vertical sliding doors. Mice were subjected to a series of 1 habituation period and 7 trials separated by 5 second intervals. The habituation period consists of 1 minute during which the mouse was left to explore the entire maze and allowed to return to the start position. At the start of a trial the door is opened and the mice have the option of entering either the left or right arm. The mice were then left in the arm of choice for 5 seconds and recorded before being allowed to return to the start box. If they did not freely return to the start box or remained stationary for 15 seconds, they are gently nudged to avoid stress of handling. Alternation rate (% alternation) and left/right (% left right ratio) of arm choice was measured. The total time was recorded on how long the animals took to complete the 7 trials. T-maze was conducted on day 7 after surgery.

4.2.8 Brain oedema

Brain water content, which we interpret as a marker of brain oedema was calculated using the wet-dry method as previously described (Gibson et al., 2005a). Brains were separated into ipsilateral and contralateral hemispheres and were gently blotted with tissue paper to remove small quantities of absorbent cerebral spinal fluid. All hemispheres were then weighed in glass vials with a basic precision scale to within 0.1 mg, giving the wet weight. Samples were placed in an oven at 80 °C for 24 h, after which they were weighed to obtain the dry weight. The percentage of

water of each sample was calculated as follows: percent brain water = [(wet weight – dry weight) / wet weight] × 100.

4.2.9 Statistical Analysis

All data are expressed as means ± standard error of the means (SEM). Survival data was analysed by Kaplan-Meier curve and Mantel-Haenszel log-rank test to identify differences. All other experiments conducted over a period of days (body weight, neurological score, foot fault and t-maze) were analysed by repeated measures analysis of variance (Genstat 16.0). Tables 4.4-4.5, 4.7-4.8 and 4.10 documents number of animals for each group at each time point for young C57 Bl/6 and BPH/2 mice. Brain water content data was analysed using two-way ANOVA for differences according to hemisphere and treatment. The data from the T-maze and foot fault conducted for only one day post-MCAO was analysed using one-way ANOVA to identify differences according to treatment. *Post hoc* analyses were carried out using either Bonferroni tests or Newman-Keuls (repeated measures). Data analysis was conducted using GraphPad Prism Version 5.0 for windows (GraphPad Software). The criterion for statistical significance was $P < 0.05$.

4.3 RESULTS

4.3.1 Adult C57 Bl/6

Initially, MCAO was induced for 60 minutes in 4 animals (2 progesterone treated and 2 vehicle treated). However, one animal treated with vehicle displayed weight loss of greater than 20% (after 72 hrs post-surgery) and one progesterone-treated animal displayed barrel rolling and loss of the

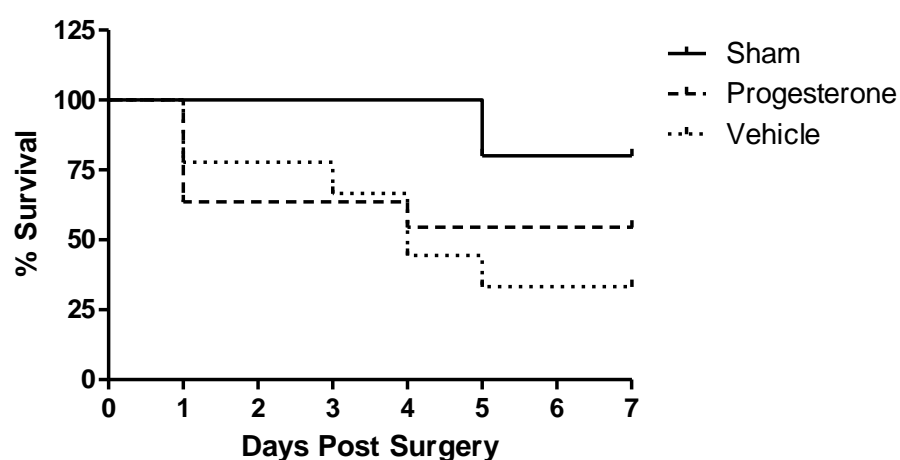
ability to self right at 1 hr post-MCAO. For welfare animals, both of these animals were sacrificed and, in an attempt to increase the welfare of animals, the occlusion time for subsequent experiments was reduced to 30 minutes.

4.3.1.1 Assessment of general well being in adult C57 BI/6

By 7 days post-surgery, one sham-operated mouse was killed due to weight loss being greater than 20% from pre-surgery weight, 6 vehicle-treated animals were killed for welfare reasons (4 animals for >20% weight loss, 2 for barrel rolling) and in the progesterone-treated group 4 animals were killed for welfare reasons (1 weight loss <20%, 3 barrel rolling) and one animal was found dead the day after surgery. Analysis of survival data revealed no significant differences in survival rate between treatment groups ($P=0.3286$) (figure 4.1). All animals lost weight for the first few days following either MCAO or sham surgery before beginning to gain weight.

Analysis of weight loss found a significant difference between animal groups [$F(2,22)=3.62$, $P=0.044$]. Newman-Keuls post-hoc analysis found both the progesterone ($P<0.05$) and vehicle-treated ($P<0.05$) groups gained weight at significantly slower rate compared to shams. However, there were no significant differences in body weight gain between the progesterone and vehicle treated groups (figure 4.2).

Figure 4.1: Comparison of post-surgery survival according to treatment/surgery of adult C57 Bl/6 mice.



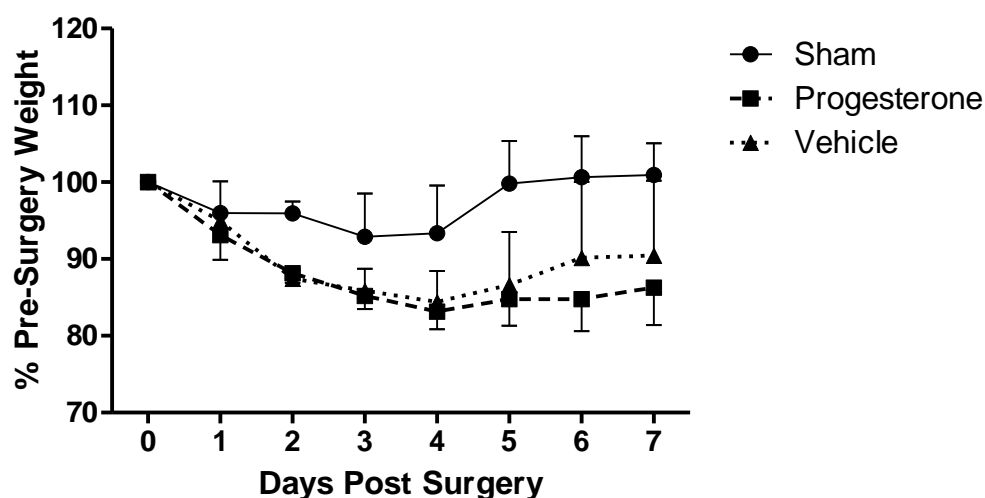
Mortality data expressed using the Kaplan-Meier curve and analysed using the Mantel-Haenszel log-rank test. There was no significant difference between groups in % of surviving animals in each group post-surgery ($P=0.3286$).

Table 4.4: Number of mice in each experimental group used for weight loss that underwent surgery for adult C57 Bl/6

Day	Number of animals per group		
	Sham	Progesterone	Vehicle
0	5	11	9
1	5	11	9
2	5	7	7
3	5	7	7
4	5	7	6
5	4	6	4
6	4	6	3
7	4	6	3

Weights for each animal were collected prior to functional outcomes or were killed for welfare reasons

Figure 4.2: Weight loss of pre-surgery weight of adult C57 Bl/6



Analysis of weight loss found a significant difference between animal groups [$F(2,22)=3.62$, $P=0.044$]. Both the progesterone ($P<0.05$) and vehicle-treated ($P<0.05$) groups gained weight at significantly slower rate compared to shams. There was no difference in body weight gain between progesterone and vehicle-treated animals groups. Data points are expressed as means \pm SEM.

Table 4.5: Number of mice in each experimental group used for behavioural testing for young C57 Bl/6

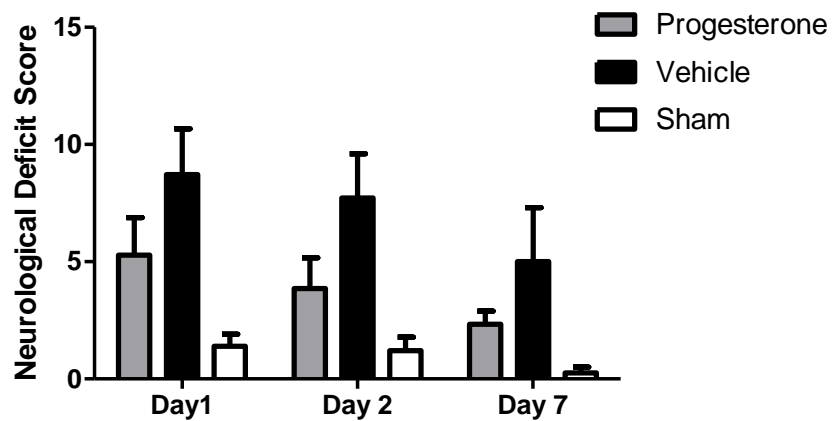
Day	Number of animals per group		
	Sham	Progesterone	Vehicle
1	5	7	7
2	5	7	7
7	4	6	3

Number of animals that underwent surgery and survived at each time point for neurological score, foot-fault and T-maze.

4.3.1.2 Neurological score for adult C57 BI/6

A significant difference was found between groups [$F(2,16)=4.93$, $P=0.021$]. Following MCAO, a significant neurological deficit was observed in both vehicle-treated ($P<0.05$) and progesterone-treated ($P<0.05$) animals in comparison to sham-operated controls. Progesterone treatment significantly reduced the neurological deficit present following MCAO compared to vehicle treatment ($P<0.05$) (figure 4.3).

Figure 4.3: Neurological deficit score for adult C57 BI/6

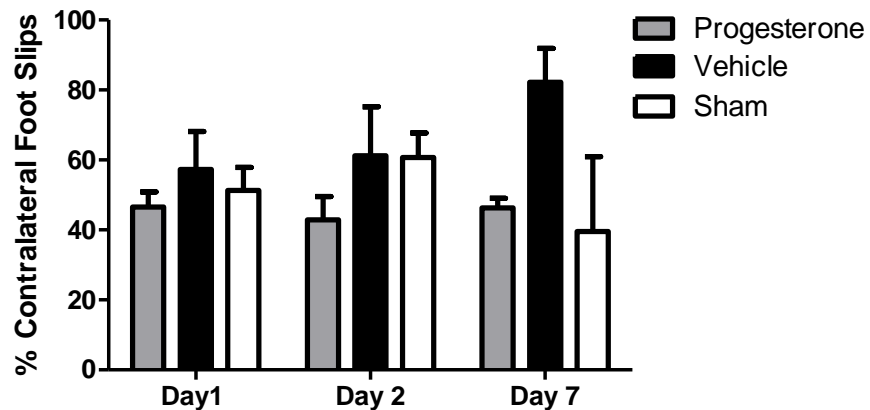


A significant difference was found between groups [$F(2,16)=4.93$, $P=0.021$]. Newman-Keuls post-hoc analysis found both vehicle-treated ($P<0.05$) and progesterone-treated ($P<0.05$) animals to have a greater neurological deficit in comparison to shams, and progesterone treatment reduced neurological deficit compared to vehicle ($P<0.05$). Data expressed as means \pm SEM.

4.3.1.3 Foot-fault for adult C57 BI/6

Unilateral foot-faults were expressed by the number of contralateral foot-faults as a percentage of the total errors made; a value of 50% represents an equal number of errors made by both sides. For example, on day 1, shams revealed no functional deficit as they made, on average, 51.27 ± 6.62 (mean \pm SEM) errors on the contralateral side, whereas, following MCAO, animals showed a greater number of contralateral foot slips. Statistical analysis found no difference in the number of contralateral foot-faults between treatment groups [$F(2,16)=1.82$, $P=0.194$] (figure 4.4).

Figure 4.4: Foot fault for adult C57 BI/6



No significant difference was found between groups [$F(2,16)=1.82$, $P=0.0194$]. Data expressed as means \pm SEM.

4.3.1.4 T-maze for adult C57 Bl/6

The T-maze is a test of spatial learning and working memory. There was no difference in T-maze performance following either MCAO surgery (in comparison to shams) or by drug treatment. The outcome measures used were: alternation rate, left/right alternation % and time taken to complete trials (table 4.6).

Table 4.6: T-maze summary for adult C57 Bl/6

Group	% Alternation	% L/R Ratio	Time taken (mins)
Sham	58.34 ± 4.81	57.14 ± 5.83	7.78 ± 0.94
Progesterone	38.89 ± 12.67	54.62 ± 14.03	6.27 ± 0.91
Control	50.00 ± 9.62	57.14 ± 14.29	5.59 ± 0.21

There was no significant difference between groups in terms of % alternation rate [$F(2,10)=0.83$, $P=0.4656$]. Left/Right alternation ratio % was found not to be significantly different [$F(2,10)=0.01$, $P=0.9865$] as well as time taken to complete trials [$F(2,10)=1.29$, $P=0.3184$]. Data expressed as means ± SEM.

4.3.1.5 Modified analyses of outcomes in adult male mice to consider death

Conventionally, as done previously in the results, functional end points for pre-clinical studies are based on data from animals which have survived to the point of observation, and due to attrition from spontaneous deaths or for welfare reasons, animals are lost and no longer contribute to data acquisition. In clinical trials, death is included as an outcome and it could be beneficial for pre-clinical studies to also include death as an end point, for example, with the modified Rankin Scale; in this respect, death is usually assigned a 'worse' value.

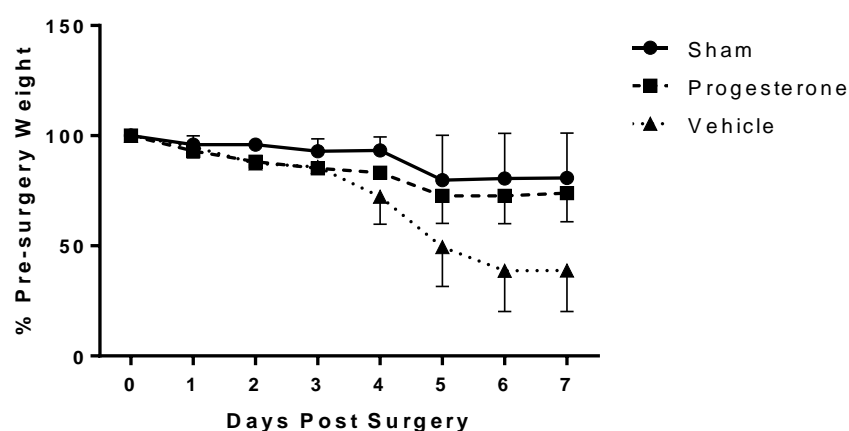
Animals killed for welfare due to excessive weight loss, were excluded from weight loss analysis from that point onwards and so weight loss data is lost. Therefore, in order to utilise these animals for analysis, animals which have died due to excessive weight loss are given a worse case value of 0% weight from pre-surgery at the point they die or killed for welfare (two animals from day 4 and one at day 5 in the vehicle treated group, one animal from day 4 in the progesterone treated group and one animal in the sham group from day 4 post-surgery). In the adjusted analysis, all animals lost weight for the first few days following either MCAO or sham surgery before beginning to gain weight. Statistical analysis found no difference between groups [$F(2,22)=2.05$, $P=0.152$] (figure 4.5).

Table 4.7: Number of mice in each experimental group used for modified weight loss analysis for adult C57 BI/6

Day	Number of animals per group		
	Sham	Progesterone	Vehicle
0	5	11	9
1	5	11	9
2	5	7	7
3	5	7	7
4	5	7	7
5	5	7	7
6	5	7	7
7	5	7	7

Weights for each animal were collected prior to functional outcomes and animals which were killed for excessive weight loss were counted and imputed as 0% weight from pre-surgery

Figure 4.5: Modified weight loss of pre-surgery weight for adult C57 BI/6

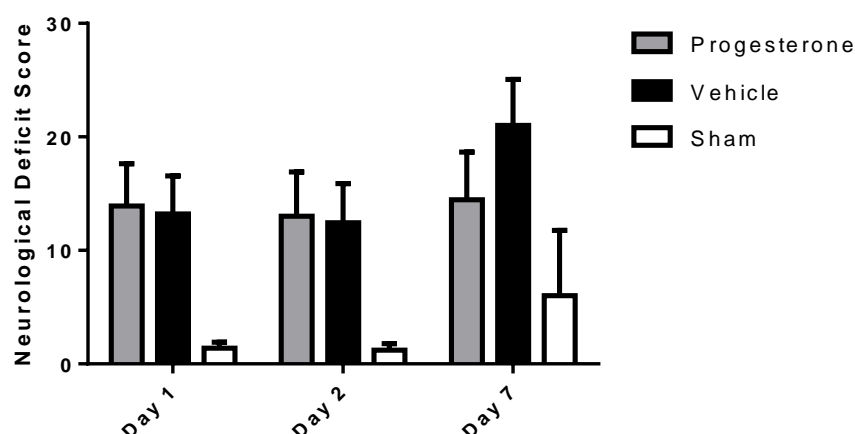


No significant difference was found between groups [$F(2,22)=2.05$, $P=0.152$]. Data points are expressed as means \pm SEM (sham $n=5$, Progesterone $n=11$, Vehicle $n=9$).

In the modified neurological deficit score analyses, animals that died prematurely were assigned a score of 29 points i.e. beyond the maximum neurological deficit possible. Animals include; one sham from day 4, four animals from day 1 and one from day 4 in the progesterone treatment group, two animals from day 2 followed by one from day 3 and two from day 4 in the vehicle treated group. No significant difference was found between animal groups [$F(2,22)= 2.72$, $P=0.088$] (figure 4.6). On day seven, sham neurological deficit score is higher compared to previous time points. This is due to one animal which was killed for weight loss. In comparison of modified to unmodified (figure 4.3) neurological deficit scores on day 7, both progesterone and vehicle scores were higher than the unmodified analysis due to deaths. Although, neurological scores in

terms of treatment were still relative to each other i.e. vehicle neurological scores were still higher versus progesterone treatment.

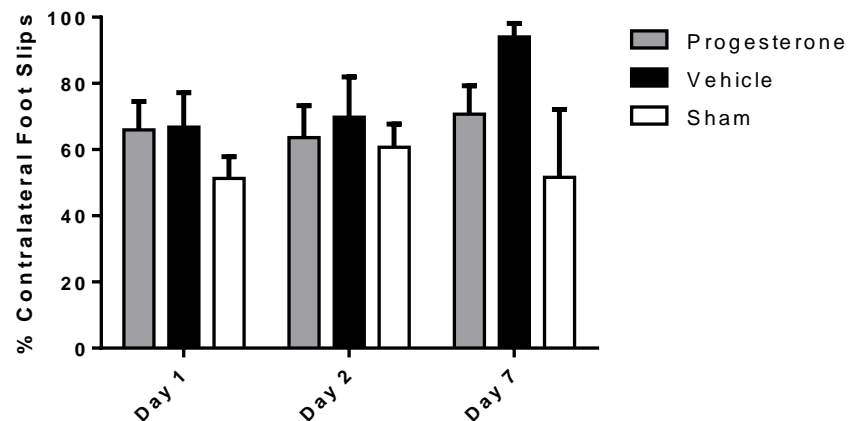
Figure 4.6: Modified neurological deficit score for adult C57 Bl/6



No significant difference was found between animal groups [$F(2,22)= 272$, $P=0.088$]. Data expressed as means \pm SEM (sham $n=5$, Progesterone $n=7$, Vehicle $n=7$).

Unilateral foot-faults were expressed by the number of contralateral foot-faults as a percentage of the total errors made; a value of 50% represents an equal number of errors made by both sides, as analysed previously with foot-faults. However, in this further analysis, animals which have been killed for welfare or died spontaneously were scored as having 100% contralateral foot faults (one sham from day 4, four animals from day 1 and one from day 4 in the progesterone treatment group, two animals from day 2 followed by one from day 3 and two from day 4 in the vehicle treated group). Statistical analysis found no difference between groups [$F(2,22)= 1.36$, $P=0.279$](figure 4.7).

Figure 4.7: Foot fault for adult C57 Bl/6 adjusted for deaths



There was no difference in contralateral foot-faults found between groups [$F(2,66)= 2.89$, $P=0.063$]. Data expressed as means \pm SEM (sham $n=5$, Progesterone $n=7$, Vehicle $n=7$).

4.3.2 Effect of aging on outcome following MCAO and progesterone treatment

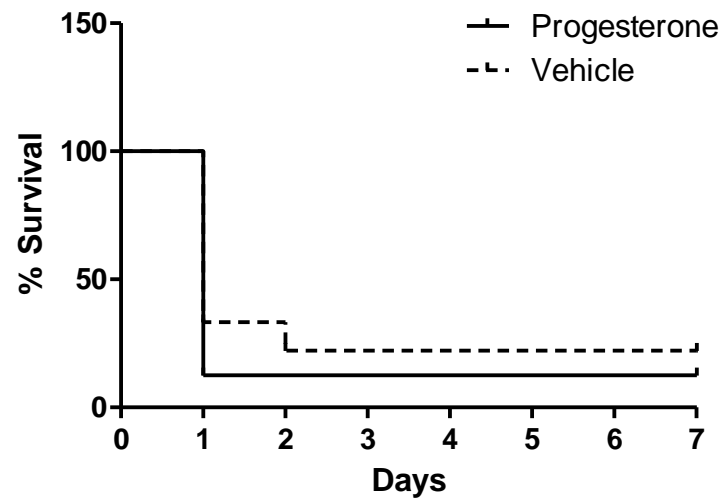
In the initial surgeries of aged C57 Bl/6 mice, a total of ten animals (five progesterone treated and five vehicle treated) were subjected to 30 minutes of occlusion. This length of occlusion was selected, as explained previously with the young C57 Bl/6 animals, to increase survival but also allow measurable functional deficits. In the progesterone treated group only one animal survived to the intended end of study, i.e. day 7 post-MCAO, one was found dead the following day after surgery, and 3 were killed for welfare reasons (barrel rolling). The vehicle-treated group comprised two animals surviving to day 7, one found dead the next day after surgery, and two animals killed for welfare (one for barrel rolling after

surgery and another on the same day for inability to recover from surgery).

Because of the number of deaths after MCAO in the aged animals, post-operative care was changed in an attempt to increase survival. All animals to this point, including young C57 Bl/6 and initial aged animals recovered at 22°C. The temperature at post-operative recovery was then increased to 28°C for 4 animals (two progesterone treated and two vehicle treated) in an attempt to increase survival by keeping animals warm following advice from the biomedical sciences unit. However, all animals were found dead the following day.

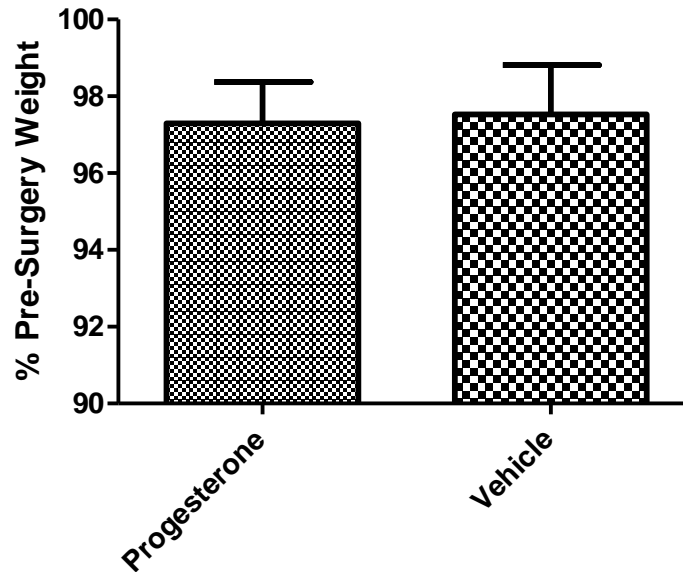
Due to the number of deaths following 30 minutes of MCAO the occlusion time was reduced to 15 minutes and the temperature during recovery was brought back down to 22°C. In total four animals underwent 15 minutes of occlusion (two progesterone-treated and two vehicle-treated animals) with one of the progesterone treated animals having adverse effects from anaesthesia and being euthanized. The remaining three animals following surgery the next day were killed for welfare due to veterinary advice (two progesterone-treated and one vehicle treated). Therefore, reducing the occlusion time from 30 to 15 minutes did not improve the survival of aged C57 Bl/6 mice. Due to the high number of deaths overall, which revealed no significant difference between treatment groups ($P=0.5266$) (figure 4.8), the data available was too limited for functional end points. Although, enough data was available at day one following surgery for weight loss and assessment of brain water content, which we interpret as a marker of brain oedema at time of death. There was no difference between treatment groups for weight loss the day after surgery or oedema at time of death ($P=0.8903$) (figure 4.9).

Figure 4.8: Comparison of post-surgery survival according to treatment/surgery of aged C57 Bl/6 mice.



Mortality data expressed using the Kaplan-Meier curve and analysed using the Mantel-Haenszel log-rank test. There was no significant difference between groups in % of surviving animals in each group post-surgery ($P=0.5266$).

Figure 4.9: Weight loss of aged C57/Bl6 24h post-MCAO

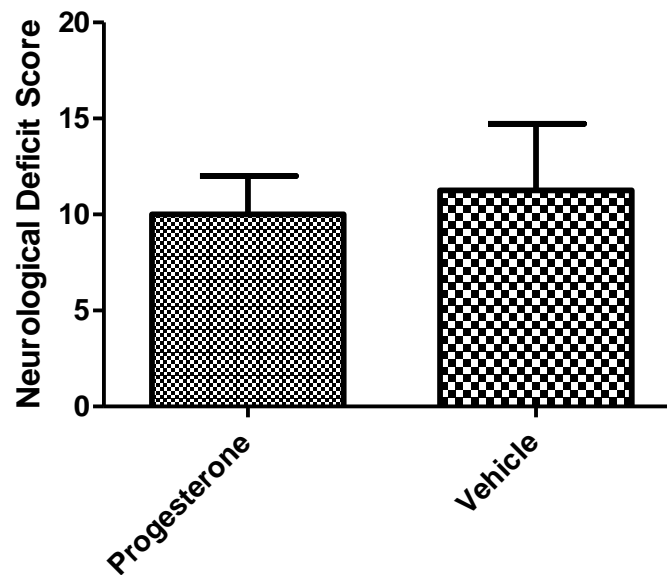


Body weight was measured the following day post surgery in % of pre-surgery weight. Analysis with t-test found no difference in body weight gain between progesterone and vehicle treated animals groups. Data points are expressed as means \pm SEM ($n=5$, $P=0.8903$).

4.3.2.1 Neurological score for aged animals

Following MCAO, no differences in neurological deficit was observed between treatment groups after 30 minutes of occlusion in aged animals (figure 4.10).

Figure 4.10: Neurological score for aged C57/Bl6 24h post-MCAO

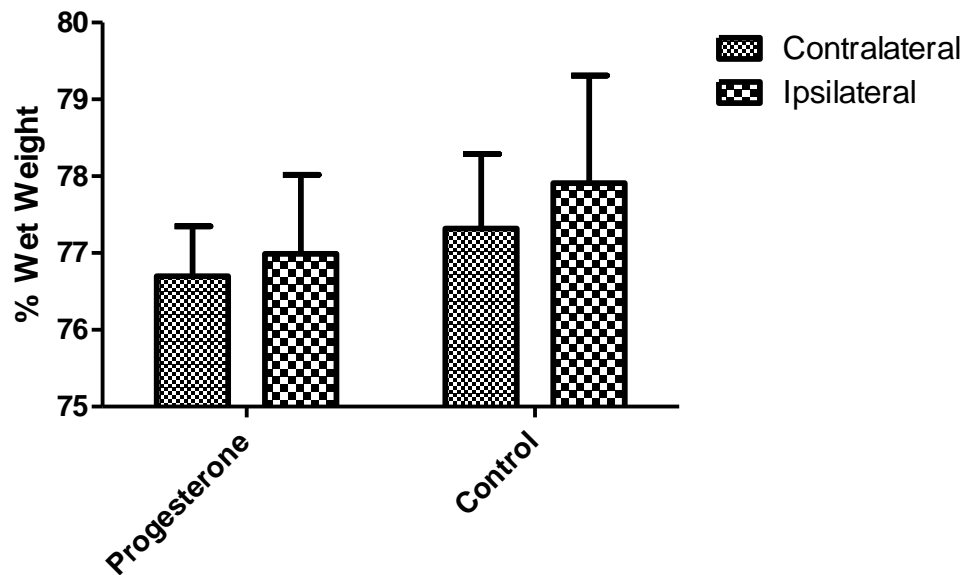


Aged animals underwent 30 minutes of MCAO and were either treated with progesterone or vehicle. There was no difference found in neurological deficit score between treatment groups. Data expressed as means \pm SEM (progesterone $n=2$, vehicle $n=4$).

4.3.2.2 Oedema at time of death for aged C57 Bl/6

Following MCAO, no difference in brain water content at time of death was observed between treatment groups after 30 minutes of occlusion in aged animals [$F(1,18)=2.486$, $P=0.1323$] (figure 4.11).

Figure 4.11: Oedema at time of death for aged C57 Bl/6



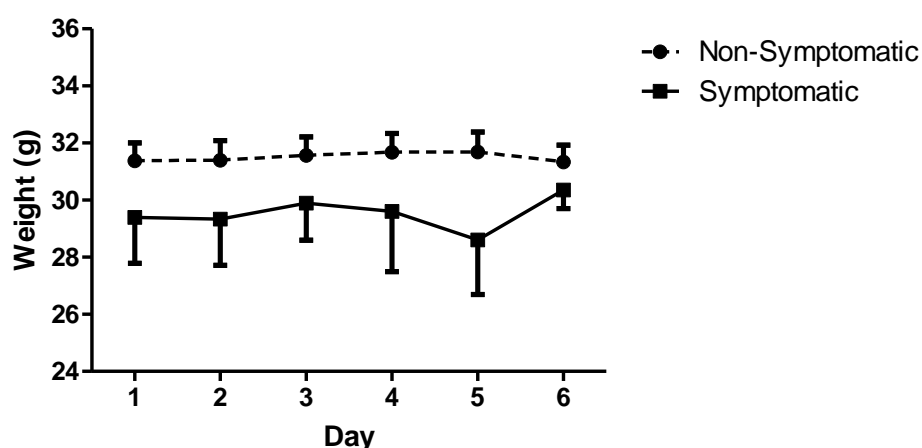
Wet-dry analysis of brain water content found MCAO in aged animals did not result in significant increase of ipsilateral brain water content regardless of treatment [$F(1,18)=2.486$, $P=0.1323$]. Data expressed as means \pm SEM (progesterone $n=4$, vehicle $n=7$).

4.3.3 Non-obese diabetic mice (NOD/ShiLtJ)

Information from the supplier of NOD/ShiLtJ mice, informed of marked decrease in pancreatic insulin content, which occurs in females about 12 weeks of age and several weeks later in males. We used male animals at 21 weeks of age and a small number were subjected to MCAO surgery and dosing to evaluate their tolerance to procedures. In total four NOD/ShiLtJ mice underwent surgery, two shams and two that underwent MCAO for 30 minutes (one treated with progesterone and one vehicle treated). The following day animals that underwent MCAO had died and one sham was killed for welfare reasons. We then used the remaining animals, initially

destined for surgery, to monitor urine glucose levels over five days (AccuBioTech Co, Newark, DE, USA). Animals were either found to be negative for glucose in their urine (non-symptomatic animals) or had $>55\text{mmol/L}$ of glucose (symptomatic animals). Symptomatic animals had a lower body weight compared to non-symptomatic animals [$F(1,53)=10.87$, $P=0.0017$] (figure 4.12).

Figure 4.12: NOD/ShiLtJ weight over time of monitoring

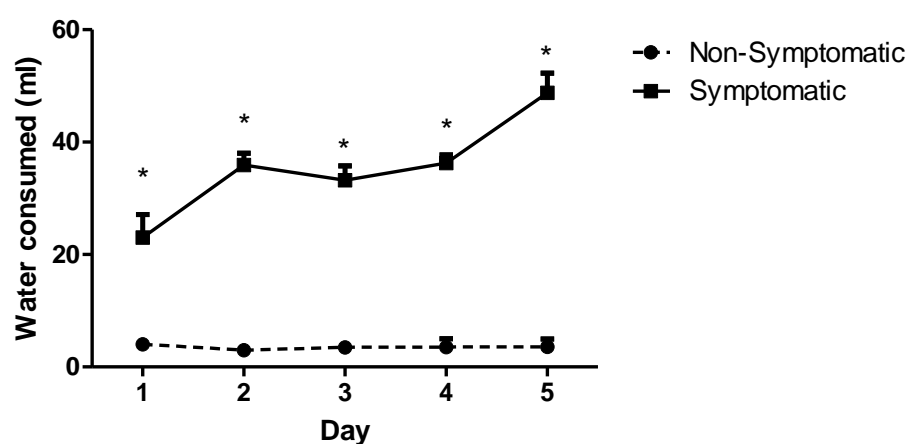


Two-way analysis of variance found symptomatic mice have significantly reduced body weights compared to non-symptomatic animals [$F(1,53)=10.87$, $P=0.0017$]. Data expressed as means \pm SEM (non-symptomatic $n=8$, symptomatic $n=3$).

Also, water consumption in both symptomatic and non-symptomatic animals were recorded every 24 hrs, for five days, by measuring water consumed from the last recording from the previous 24 hrs. Symptomatic animals had extremely elevated water consumption compared to non-

symptomatic animals, which increased over time of monitoring [$F(1,44)=1772$, $P<0.0001$] (figure 4.13).

Figure 4.13: NOD/ShiLtJ water consumption over time of monitoring



Two-way analysis of variance found symptomatic mice have significantly reduced body weights compared to non-symptomatic animals [$F(1,44)=1772$, $P<0.0001$] (non-symptomatic $n=8$, symptomatic $n=3$). Bonferroni post-hoc analysis found symptomatic animal have significantly reduced body weights compared to non-symptomatic animals at each time point ($P<0.0001^*$).

Due to symptomatic animals having a high water demand, it is unlikely they would have survived when challenged with surgery. This is because animals recovering straight after surgery are less inclined to drink and fluid replacement would be insufficient for symptomatic animals. Also, non-symptomatic animals had no signs of diabetic symptoms and so could not be used to test for the effects of diabetes on progesterone neuroprotection.

Animals were then killed via cervical dislocation and their blood was tested for the presence of ketones. Symptomatic mice were found to have >27mmol/L of ketones in their blood and non-symptomatic animals were negative for ketones (Precision Xtra, Abbot Laboratories, CA, USA).

4.3.4 Hypertensive BPH/2 mice

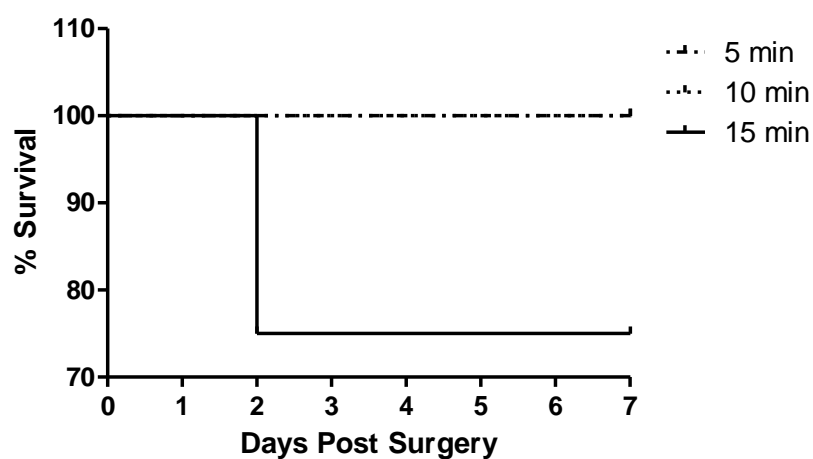
4.3.4.1 Hypertensive BPH/2 with increasing occlusion times

Hypertensive BPH/2 mice received increasing periods of MCAO in order to identify the length of occlusion required to cause measurable functional deficit without excessive mortality and weight loss. In total eight animals underwent transient MCAO (two animals for 5 minutes, two for 10 minutes and four for 15 minutes of MCAO). One animal that underwent occlusion for 15 minutes of occlusion died on the second day post surgery (table 4.8). Analysis of survival found no significant difference between different occlusion times ($P=0.6065$) (figure 4.14). All animals lost weight following surgery, but no significant difference was found between groups [$F(2,5)=0.12$, $P=0.886$] (figure 4.15).

Table 4.8: Number of BPH/ 2 mice in each experimental group used for increasing occlusion times

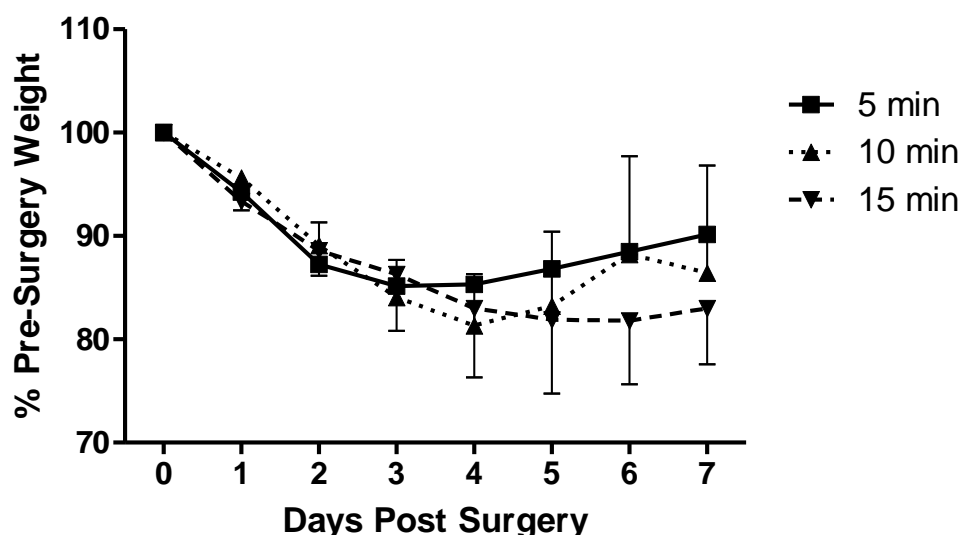
Day	Number of animals per group		
	5 minutes MCAO	10 minutes MCAO	15 minutes MCAO
0	2	2	4
1	2	2	4
2	2	2	4
3	2	2	3
4	2	2	3
5	2	2	3
6	2	2	3
7	2	2	3

Figure 4.14: Survival of BPH/ 2 hypertensive mice with increasing occlusion time



Mortality data expressed using the Kaplan-Meier curve and analysed using the Mantel-Haenszel log-rank test. There was no significant difference between groups in % of surviving animals in each group post-surgery ($P=0.6065$).

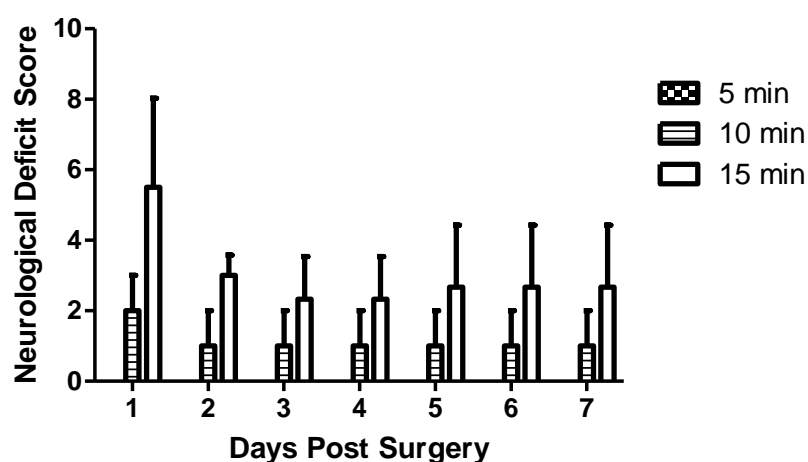
Figure 4.15: Weight Loss from pre-surgery weight of BPH/ 2 hypertensive animals with increasing occlusion time



There was no difference in body weight gain between progesterone and vehicle treated animals groups [$F(2,3)= 0.12$, $P=0.886$]. Data points are expressed as means \pm SEM.

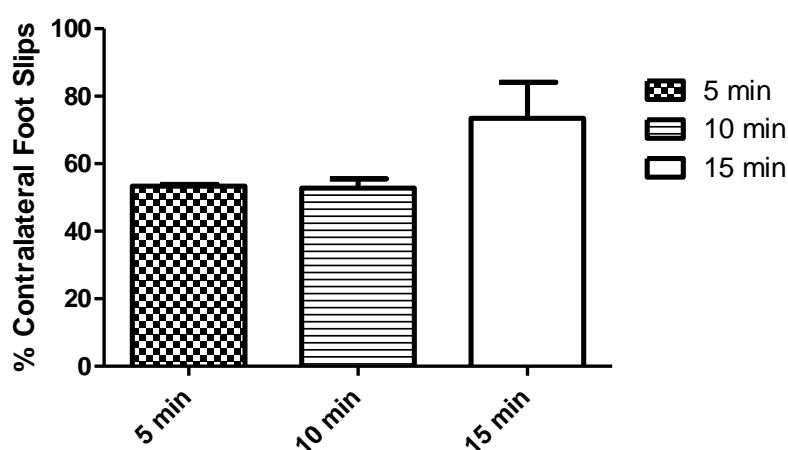
The length of occlusion was found not to effect neurological deficit [$F(2,29)=7.5$, $P=0.360$] (figure 4.16). There was no difference in contralateral foot slips between groups in the foot fault test at 24 hrs [$F(2,5)=1.50$, $P=0.3085$] (figure 4.17). The % alternation rate [$F(2,4)=0.59$, $P=0.5948$], Left/Right alternation ratio % [$F(2,4)=1.43$, $P=0.3403$] and time taken to complete trials [$F(2,4)=4.30$, $P=0.1008$] for T-maze were not significantly different between groups (table 4.9). There was no difference between treatment groups in oedema formation for animals, which survived to the seventh day [$F(2,8)=0.77$, $P=0.4954$] (figure 4.18).

Figure 4.16: Neurological deficit score for BPH/2 with increasing occlusion times



The length of occlusion was found not to effect neurological deficit [$F(2,5)=1.26$, $P=0.360$]. Data expressed as means \pm SEM.

Figure 4.17: Foot fault for BPH/2 with increasing occlusion times at 24 hrs

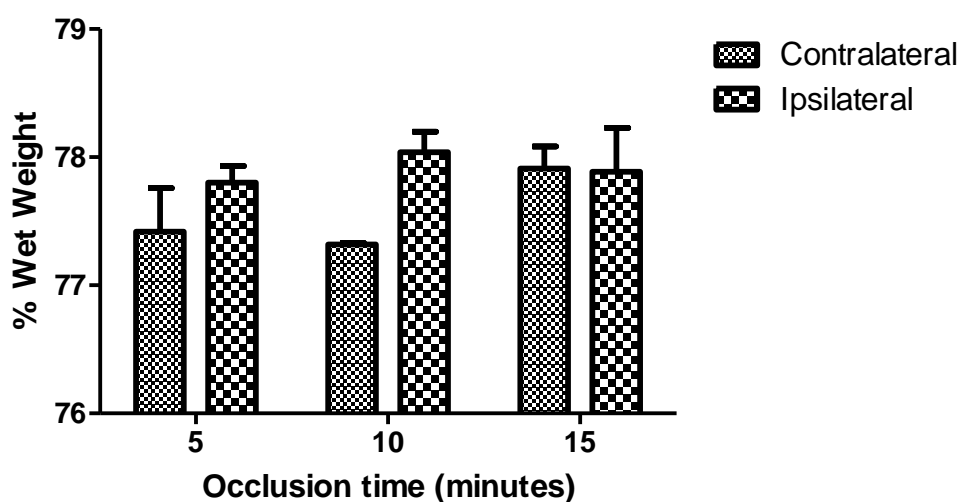


Analysis with one-way ANOVA found no significant difference in contralateral foot slips between groups [$F(2,5)=1.50$, $P=0.3085$]. Data expressed as means \pm SEM.

Table 4.9: T-maze for BPH/2 with increasing occlusion times

Group	% Alternation	% L/R Ratio	Time taken (mins)
5 Minutes	58.33 \pm 25.00	35.72 \pm 21.43	3.71 \pm 0.58
10 Minutes	58.34 \pm 8.34	64.29 \pm 7.15	2.95 \pm 0.36
15 Minutes	33.33 \pm 19.25	71.43 \pm 14.29	5.59 \pm 0.76

Analysis with one-way ANOVA found no significant difference between groups in terms of % alternation rate [$F(2,4)=0.59$, $P=0.5948$]. Left/Right alternation ratio % was also not found to be significantly different [$F(2,4)=1.43$, $P=0.3403$], as well as time taken to complete trials [$F(2,4)=4.30$, $P=0.1008$]. Data expressed as means \pm SEM.

Figure 4.18: Oedema of animals receiving increased occlusion that survived to day 7

Wet-dry analysis of brain water content found ipsilateral brain water content was not affected by time of occlusion [$F(2,8)=0.77$, $P=0.4954$].

Although not significant, due to the small number of animals, these findings indicate out of all the occlusion times analysed, 15 minutes of MCAO may be adequate to cause measurable functional deficit without excessive mortality and weight loss. Analysis of survival found no significant difference, although there was one death at 15 minutes occlusion and so longer occlusion times may result in increased mortality. There was no significant difference in weight loss found. However, at 15 minutes of occlusion, animals did lose the most weight compared to shorter occlusions times despite not being significant. Neurological deficit scores were higher following 15 minutes occlusion despite not being significant. Foot fault analysis found no significant difference between occlusion times, despite animals receiving 15 minutes of occlusion had increased contralateral foot slips, indicating greater asymmetry. T-maze analyses also found no difference between groups. Although, at 15 minutes of occlusion, animals did alternate the least, have higher tendency to turn left (contralateral to damaged side) and took the most time to complete the trials. There was no wet-weight difference found between animals with increasing times of occlusion that survived to day 7 after surgery. One animal that received 15 minutes of occlusion died spontaneously after functional tests on the second day after surgery and was excluded from oedema analysis. Oedema measurement for this animal was 77.31% contralateral and 80.65% ipsilateral wet weight.

It is likely that, despite having the most weight loss, neurological deficit score, contralateral foot slips, time taken to complete T-maze and least alternation rates, there were not enough power from animal numbers for these analyse to show a significant effect. However, there were limited animal numbers and some were reserved for dosing with progesterone.

After an adequate occlusion length was determined, the effect of progesterone in MCAO could be determined in hypertensive animals.

4.3.4.2 Progesterone in hypertensive BPH/ 2 animals and MCAO

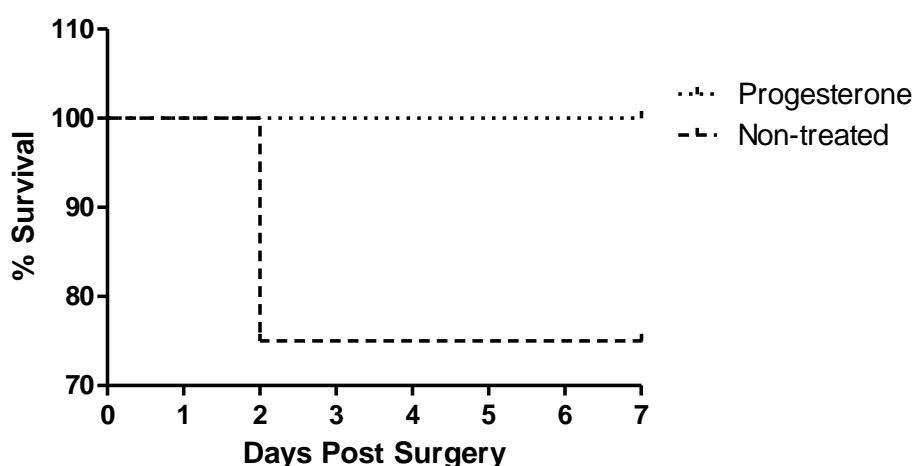
Because of limited animal numbers available, the remaining animals were assigned to be dosed with progesterone. Animals that previously underwent 15 minutes of occlusion, which were non-treated, would act as a comparison group. Ideally animals would be injected with vehicle but this was not possible due to the number of animals remaining after evaluating occlusion times. A total of five animals receive MCAO for 15 minutes and were dosed with progesterone. These were compared with non-treated animals that received 15 minutes of MCAO. All progesterone treated animals survived to the end of all functional end-points on day seven (table 4.10).

Table 4.10: Number of BPH/ 2 mice in progesterone treated and non-treated experimental group

Day	Number of animals per group	
	Progesterone	Non-treated
0	5	4
1	5	4
2	5	4
3	5	3
4	5	3
5	5	3
6	5	3
7	5	3

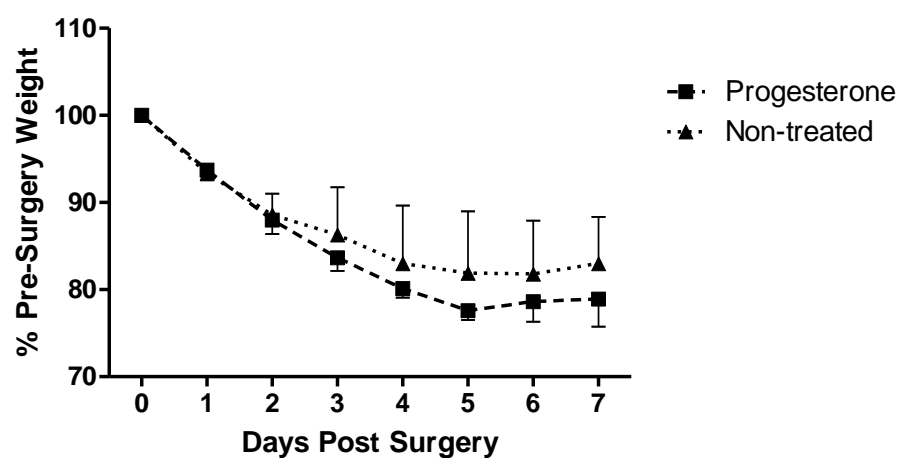
Analysis of survival found no significant difference between progesterone and non-treated animals ($P=0.2636$) (figure 4.19). All animals lost weight following surgery, but no significant difference was found between groups [$F(1,7)= 0.58$, $P=0.472$] (figure 4.20). Neurological deficit analysis found progesterone treated mice not to differ significantly in neurological deficit in comparison to non-treated animals [$F(1,7)=3.62$, $P=0.099$] (figure 4.21). There was no difference in contralateral foot slips between groups in the foot fault test ($P=0.0679$) (figure 4.22). Analysis of t-maze tasks found no difference in treatment groups in % alternation rate [$F(1,7)= 0.31$, $P=0.600$] (4.23), but did find progesterone treatment to lower Left/Right alternation ratio % [$F(1,6)=9.35$, $P=0.022$] (figure 4.24) and reduce the time taken to complete trials for T-maze [$F(1,6)=16.44$, $P=0.007$] (figure 4.25). There was no difference found in oedema between treatment groups [$F(1,12)=1.23$, $P=0.2884$] (figure 4.26).

Figure 4.19: The percentage of BPH/2 animals that survived post surgery over time.



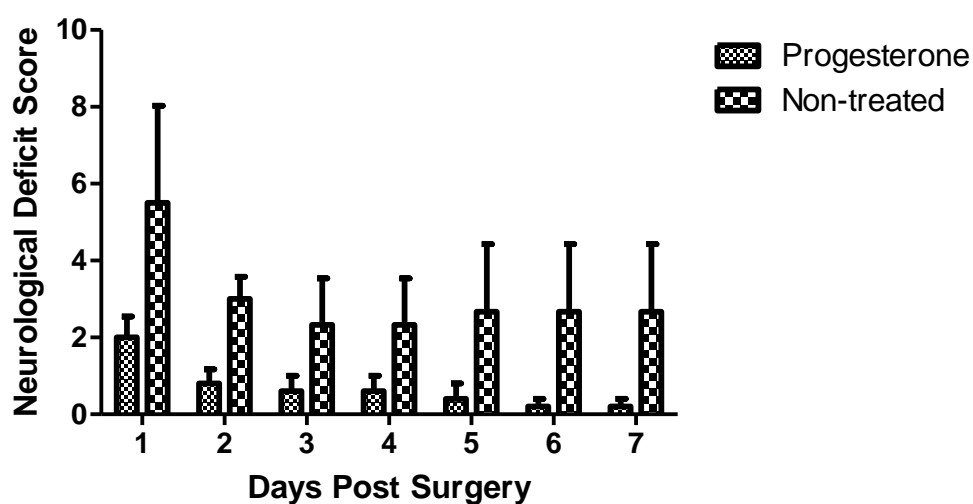
Mortality data expressed using the Kaplan-Meier curve and analysed using the Mantel-Haenszel log-rank test. There was no significant difference between groups in % of surviving animals in each group post-surgery ($P=0.2636$).

Figure 4.20: Weight loss from pre-surgery weight of BPH/2



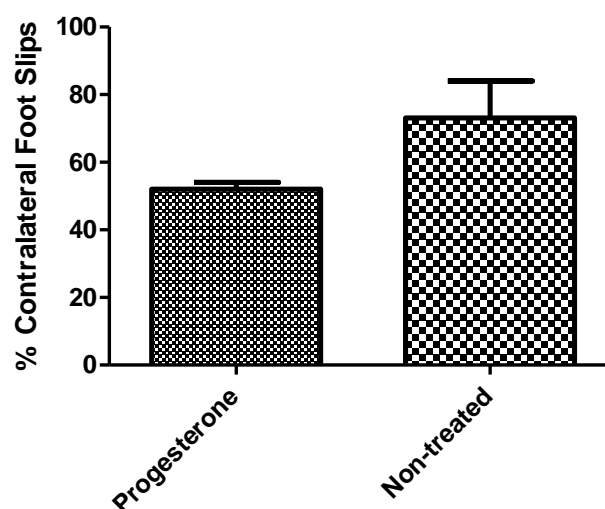
All animals significantly loss weight after surgery, but there was no difference in body weight gain between progesterone and vehicle treated animals groups [$F(1,7)= 0.58$, $P=0.472$]. Data points are expressed as means \pm SEM.

Figure 4.21: Neurological score for BPH/2



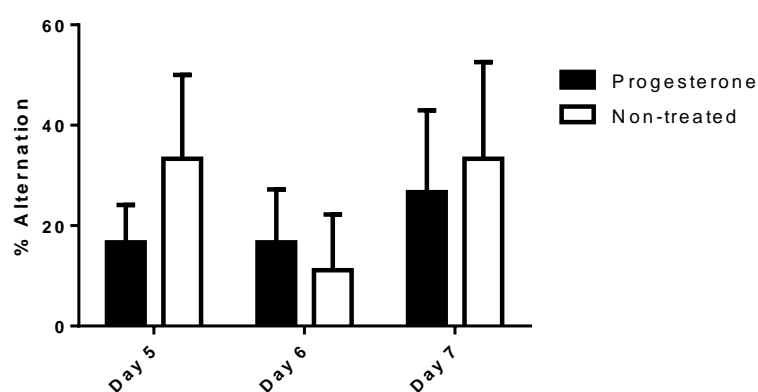
Progesterone treatment reduced neurological deficit compared to controls [$F(1,7)=3.62$, $P=0.099$]. Data expressed as means \pm SEM.

Figure 4.22: Foot fault for BPH/2



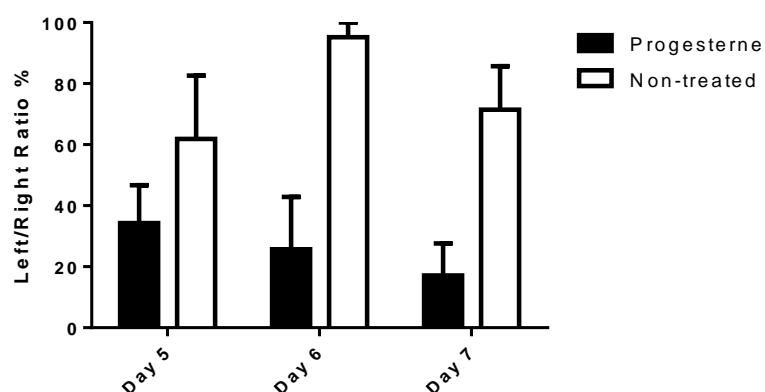
Contralateral foot-faults for day (%) the day following surgery. There was no difference found between progesterone and non-treated ($P=0.0679$). Data expressed as means \pm SEM.

Figure 4.23: T-maze % alternation for BPH/2



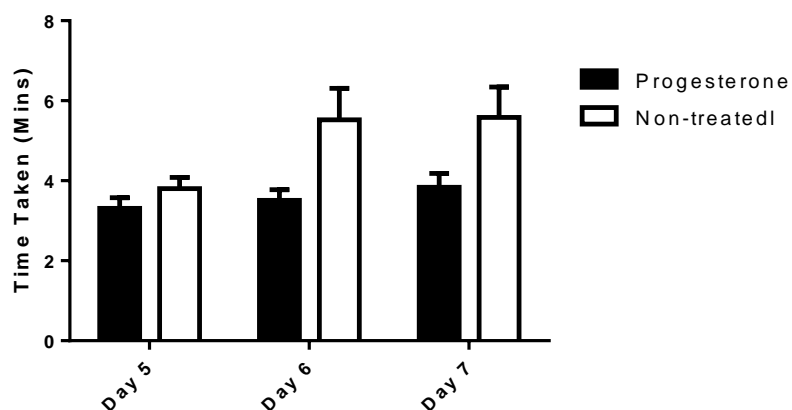
T -maze % alternation on day 5 to 7. There was no difference found between progesterone and non-treated groups [$F(1,7)= 0.31$, $P=0.600$]. Data expressed as means \pm SEM.

Figure 4.24: T-maze % left/right ratio for BPH/2



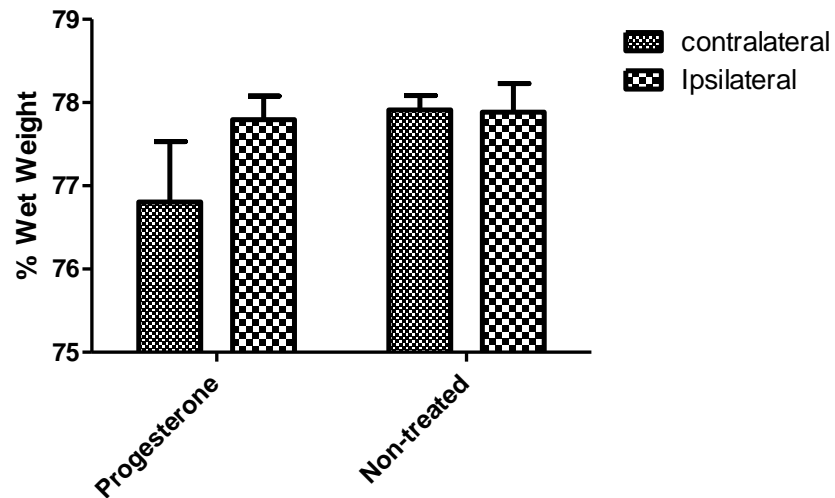
Progesterone treated group significantly turns left greater than vehicle treated groups [$F(1,6)=9.35$, $P=0.022$]. Data expressed as means \pm SEM.

Figure 4.25: T-maze for time taken to complete trials for BPH/2



Time taken to complete T-maze spontaneous alternation trials on days 5-7. Non-treated animals took longer to complete t-maze than progesterone treated animals [$F(1,6)=16.44$, $P=0.007$]. Data expressed as means \pm SEM.

Figure 4.26: Oedema of BPH/2 that survived to day 7

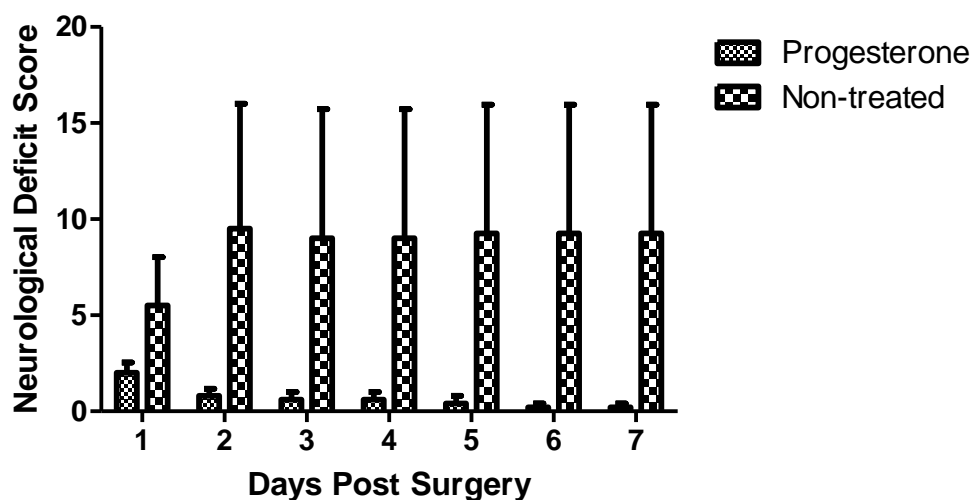


Wet weight measured at the end of day 7 after behavioural tests. There was no difference in wet weight between progesterone and non-treated groups [$F(1,12)=1.23$, $P=0.2884$]. Data expressed as means \pm SEM.

4.3.4.3 Modified analyses of outcomes in hypertensive BPH/2 mice to consider death

A modified 29-point neurological deficit score was used to encompass animals which have died, including interventions for welfare, as mentioned earlier with modified neurological score for young C57 Bl/6 mice. Animals that have died are given 29 points to represent worse possible outcome in analysis. Following MCAO, progesterone treatment did not significantly reduce neurological deficit [$F(1,7)= 2.23$, $P=0.179$] (figure 4.27).

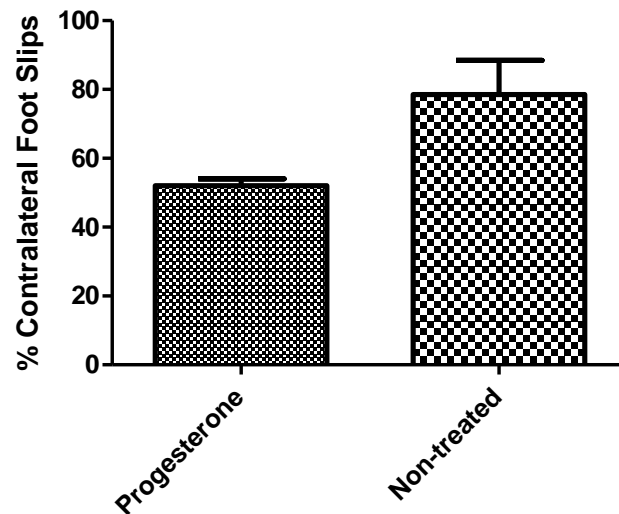
Figure 4.27: Modified neurological deficit Score for BPH/2



Progesterone treated animals did not reduce function deficit compared to non-treated animals [$F(1,7)= 2.23$, $P=0.179$]. Data expressed as means \pm SEM (sham $n=5$, Progesterone $n=5$, Non-treated $n=4$).

Unilateral foot-faults were expressed by the number of contralateral foot-faults as a percentage of the total errors made; a value of 50% represents an equal number of errors made by both sides, as analysed previously with foot-faults. However, in addition, animals which have been killed for welfare or died spontaneously were scored as having 100% contralateral foot faults (figure 4.28). Progesterone dosed animals had less contralateral foot slips compared to non-treated animals ($P=0.0313$).

Figure 4.28: Modified foot fault for BPH/2



Percentage contralateral foot faults for day 1 post surgery. Animals which have died are imputed as having 100% contralateral foot slips. Progesterone was found to reduce contralateral foot slips ($P=0.0313$). Data expressed as means \pm SEM.

4.4 DISCUSSION

The current study shows that sustained delivery of progesterone in adult C57 Bl/6 mice, initiated following the onset of transient MCAO, is beneficial in terms of promoting functional recovery. Progesterone treatment resulted in an improvement in the neurological deficit, but not a reduction in the number of contralateral foot-faults. Following MCAO, there was a clear neurological deficit and this deficit was reduced following progesterone treatment. In the present study, our outcomes have focused on functional measures, as clinical trials of novel stroke therapies use functional measures as their primary end-point. In humans, the size of the lesion from stroke does not always correlate well with functional

impairment (Pineiro et al., 2000). It is important for pre-clinical studies to have functional measures relevant to stroke outcomes in patients, as a major goal for the treatment of stroke is for patients to be functionally independent and so pre-clinical studies need to reflect the importance of functional outcomes. Restoration of behavioural function needs to be demonstrated in the pre-clinical evaluation of any putative stroke therapy. However, pre-clinical studies vary in terms of species, age, sex and doses that may be contributing factors to variations in progesterone's apparent efficacy as a neuroprotectant.

Previous studies have also reported the ability of progesterone to improve functional deficit, as measured by neurological score following MCAO (Chen and Chopp, 1999; Coomber and Gibson, 2010; Gibson et al., 2011; Jiang et al., 2009; Kumon et al., 2000; Wang et al., 2010). However, these have tended to use a much simpler scale, which simply grades function by 5 (rats) or 6 (mice) levels, ranging from 'normal' to 'severely impaired' (Hayakawa et al., 2008; Longa et al., 1989). In the current study, we utilised a 28-point neurological deficit score, first developed by Clark *et al* 1998 (Clark et al., 1998), which is specifically designed for assessment following focal cerebral ischaemia and is a more sensitive measure. Interestingly, when using a modified 29-point version that included death as a worse outcome, progesterone was not beneficial compared to vehicle. This shows that including animals that died, by giving them a worse score than the maximum in analysis can influence outcome.

Functional deficits, as measured by the foot-fault test found no change in the number of contralateral foot-faults between progesterone and vehicle treatment. Also, the percentage of contralateral foot-faults, in both vehicle and progesterone treatment groups, were not found to be different to shams. This could be attributed to the occlusion time, which was 30

minutes in this study. Other mouse studies tended to use longer occlusion times and would result in greater functional deficits being maintained over a longer time period (Coomber and Gibson, 2010; Gibson et al., 2005b; Gibson et al., 2011; Gibson and Murphy, 2004). However, we chose 30 minutes in an attempt to increase survival following MCAO. As with the neurological score, death was considered in a modified analysis and progesterone was found not to be reduce contralateral foot slips. The consideration of death in analysis can affect outcome. However, care must be taken when you consider death in analysis, as the value of death can be arbitrary (in this case 100% foot slips for death) and a high-death rate can influence the outcome quite dramatically. Other behavioural tests, which are relevant to measuring functional deficits, include rotarod and water maze, have been used in experimental stroke previously (Gibson and Murphy, 2004). However, due to time constraints, it was not possible to include these behavioural tests in this investigation.

Stroke commonly affects cognitive processes and T-maze spontaneous alternation has been shown to involve cognitive elements of behaviour, including spatial learning and working memory (Gerlai et al., 1998). This behavioural test allows the detection of potential left or right spatial neglect and enables unchallenged locomotion as well as exploratory motivation to be observed (Gerlai et al., 1998). Studies examining neurological deficits in mice, rats and gerbils have reported a lower alternation rate in animals with focal ischaemia (Gerlai et al., 2000; Ishibashi et al., 2003b; Itoh et al., 1993; Kadam et al., 2009; Matchett et al., 2007). Animals with ischaemic brain injury have a tendency, to turn ipsilaterally towards the damaged side during the T-maze and tests of behavioural asymmetry (Carloni et al., 2008; Zhang et al., 2002). However, in this current study, there was no difference in the alternation

rate, goal arm preference or time to complete the t-maze due to surgical or drug intervention. This implies that a 30 min period of transient focal cerebral ischaemia did not produce a sufficient neurological deficit to impact upon the ability to accomplish the T-maze test.

Behavioural deficits have multiple causes, and their severity is influenced by the size and location of the stroke (Pineiro et al., 2000), extent of cortical involvement, plasticity and genetic backgrounds of outbred strains (Kadam et al., 2009). Spontaneous recovery is also known to occur in rodent brains following local trauma and small degrees of irrecoverable tissue damage. The reduced occlusion time employed in this study, as compared to others, may have left intact areas of the brain associated with exploratory behaviour and working memory. Although the hippocampus is not directly affected by transient MCAO (since it is not directly perfused by the middle cerebral artery (Hunter et al., 1998), there is evidence to suggest that, over time, it can become compromised due to decreased collateral blood flow (Tang et al., 2012).

Some studies have found progesterone to aid survival after ischaemia (Gibson and Murphy, 2004), while others have reported no effect (Alkayed et al., 2000; Coomber and Gibson, 2010; Gibson et al., 2011). However, many studies do not report survival. In the present study, no difference in survival was found between treatment groups for either animals that died or were euthanized for welfare. Weight loss has been monitored in previous studies of progesterone with some showing reduced weight loss (Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000) and others finding no significant difference (Chen and Chopp, 1999; Coomber and Gibson, 2010; Gibson et al., 2011). In the present study, progesterone and vehicle-treated MCAO groups gained weight at a reduced rate compared to shams, with no difference found between progesterone and

control groups. However, care must be taken when interpreting weight loss, as some animals were not included in the analysis, because they were killed following unacceptable weight loss from a welfare perspective (one animal in the sham group, four in the vehicle and one in the progesterone group). The modified analysis of weight loss, which considered these animals, found progesterone not to be beneficial.

We reported previously, that progesterone delivered via i.p. injection has a very short half-life in both plasma and brain (Wong et al., 2012), although this dosing method has been successful in providing neuroprotection in young male mice (Gibson and Murphy, 2004). There have been only a few studies reported involving progesterone and experimental stroke using infusion delivery in rats (Alkayed et al., 2000) and mice (Coomber and Gibson, 2010). In both studies, animals were treated with progesterone prior to MCAO unlike in the present study, the only one to date in which progesterone has been delivered via mini-pump infusion after occlusion. Our previous pharmacokinetic study of progesterone infusion with mini-pumps achieved high sustained progesterone concentrations in the brain (Wong et al., 2012). The current study demonstrates this method of dosing to be beneficial in terms of neurological deficits but not regarding indicators of general well-being (survival and weight loss), motor function or cognitive performance in the T-maze. Lack of protection in some aspects, but not others, may be due to either a dose effect or the length of initial occlusion. Increasing the progesterone dose might not necessarily result in greater neuroprotection. Some studies have used higher doses of progesterone ranging from 10-32 mg/kg via i.p. injections but found no greater beneficial effects (Chen and Chopp, 1999; Murphy et al., 2002; Murphy et al., 2000). Chen *et al* found 8 mg/kg but not 4 mg/kg or 32 mg/kg to be neuroprotective (Chen and Chopp, 1999), indicating a

relatively narrow dose-response window. Clearly it will be important to determine, in future experiments, whether there is a similar narrow therapeutic window when progesterone is delivered by loading dose and mini-pump infusion, avoiding the peaks and troughs in tissue concentrations produced by repeated injections.

Continuous progesterone administration has been shown to produce enhanced benefit in traumatic brain injury (TBI) as compared to repeated injections in a rat model (Cutler et al., 2006a). This may also be the case with progesterone in stroke and a continuous dosing method would allow tapering off progesterone to be more easily achieved. Also, clinical trials investigating the neuroprotective properties of progesterone following TBI have shown the hormone to be beneficial and safe (Wright et al., 2007; Xiao et al., 2008). In these trials, steady-state serum concentrations of progesterone were achieved, following continuous intravenous infusion in patients with acute moderate to severe TBI injury (Wright et al., 2005). Any future clinical trial evaluating neuroprotection of progesterone in stroke would need to use a method of dosing that achieved a steady state concentration lasting several days, e.g. infusion or long-acting vaginal/rectal administration.

In humans, age is a predictor of worse outcome following stroke. This investigation has also shown this to be true in aged animals. Aged animals were very susceptible to MCAO, even when occlusion time was reduced. The majority of aged animals were found dead or killed for welfare reasons the following day after surgery. Mortality is expected to be higher in older animals compared to younger ones. In rats, mortality immediately post-stroke was found to be low in young animals (6%, 3-4 months) but high in old rats (44%, 18-20 months) (Wang et al., 2003). In this investigation, the majority of deaths were due to animals being killed for welfare reasons

(60%), rather than animals found dead. The majority of animals that were found dead (66.67%) could be attributed to increased temperature in the recovery phase. Increase of temperature in recovery after MCAO seems detrimental to animals, as all mice were found dead the following day in these conditions. Temperature can affect outcomes, following stroke. Higher temperatures following ischaemic stroke is thought to accentuate the ischaemic mechanism (Zaremba, 2004), which is probably the case here. Other studies involving animal models have investigated the effects of temperature and found hypothermia in the first 24 hours after stroke onset improves stroke outcomes (Barber et al., 2004). A meta-analysis of animal studies involving hypothermia and stroke found hypothermia improved outcomes by about a third (van der Worp et al., 2007).

Survival analysis found no significant difference between treatment groups. Due to the number of deaths, because of welfare reasons or otherwise, data was limited to the first day following surgery for functional outcomes and oedema at death. Because there were no available sham animals, it was not known if the source of deaths were directly due to the surgery or from occlusion. Also, not enough data was available for both treatments groups for foot-fault analysis, as animals that were killed for welfare reasons under veterinary advice were not approved to undergo further behavioural tests. Analysis of weight, neurological deficit score and oedema found progesterone not to be beneficial. Although, due to limited data from animals in a narrow time frame, this is difficult to determine if this is the case, or rather, neuroprotection would manifest later in animals that survive.

Animals in this study are in the region of 15-16 months, which is a phase leaving middle to old age. Middle age, in both mice and rats, is considered to be 10-15 months and old age is considered to be 18-24 months. In the

upper limit of 14-15 months of middle age, most biomarkers relevant to ageing may not have changed to their full extent and some have yet to manifest (Flurkey K, 2007). A limited number of studies have investigated neuroprotection of progesterone in this age range in rodents, but mostly in animals treated prior occlusion. Toung *et al* found female reproductively senescent rats, 14-18 months of age, did not benefit from pre-MCAO treatment with progesterone (Toung *et al.*, 2004), while Alkayed *et al* using 16 month old female rats found progesterone to be beneficial when administered prior MCAO (Alkayed *et al.*, 2000). Studies have not been previously performed in aged mice that are as old as the ones investigated here. Although, Gibson *et al* did use 12 months old female reproductively senescent mice that were pre-treated with progesterone before occlusion and was found to be protective (Gibson *et al.*, 2011). Presently only one study has administered progesterone, post-onset to occlusion in old animals. This study by Wang *et al*, found progesterone to improve outcomes in 24 month old male rats (Wang *et al.*, 2010). Studies of progesterone and experimental stroke involving aged animals are limited, particularly for the dosing of animals after the start of occlusion. Age is an important factor in the outcome of stroke and further investigation is required to determine the efficacy of progesterone in aged animals. Also, this study has failed to take into account gender. It would be useful to consider, that whilst pre-menopausal females represent the population group with the lowest risk of stroke, post-menopausal females represent the group at higher risk (Appelros *et al.*, 2009). Therefore, further investigation is needed in aged female animals.

The mortality of NOD/ShiLtJ animals in the preliminary surgeries was high. The remaining animals were monitored and tested for the development and severity of diabetic symptoms found only a few animals to have high

glucose in their urine and considered to be symptomatic of diabetes. High ketones in blood, which is an indicator of type 1 diabetes at the end of monitoring, helped to confirm this. Monitoring of symptomatic mice found reduced weight compared to non-symptomatic mice, which was not surprising, as NOD mice are a model of spontaneous type one diabetes and is known to be susceptible to weight loss at the onset, similar to patients that develop the condition (Kikutani and Makino, 1992). Also, symptomatic NOD mice had increased thirst, which is characteristic for the onset of symptoms (Zhao et al., 2008). Although, this strain has characteristics of type-1 diabetes, they are problematic for the use in experimental stroke. The development of diabetes in these animals can be abrupt and age dependent, which can vary between animals. Therefore, constant monitoring is required and the number of animals that can undergo surgery at any one time is limited. Also, the excessive thirst found in this investigation for symptomatic mice makes them unlikely to survive post-surgery due to their demands in water intake. Other studies have managed to use other diabetic rodent strains to investigate experimental stroke, including type-1 (Toung et al., 2000) and type-2 diabetic strains (Tureyen et al., 2011; Vannucci et al., 2001).

Initial experiments on BPH/2 mice were to determine an adequate length of occlusion for functional outcomes. There was no one test of functional outcome that showed significance, most likely due to the small number of animals available, but occlusion of 15 minutes caused greater functional deficit in tests, without excessive mortality and weight loss. Although not significant, animals with increasing occlusion times had lower alternation rates and took longer to complete the t-maze trials, which concurs with previous work in stroke animals (Gerlai et al., 2000; Ishibashi et al., 2003a, b; Itoh et al., 1993; Kadam et al., 2009; Matchett et al., 2007).

However, increased occlusion times resulted in increased tendency to favour the left goal arm (contralateral to damaged side), which is contrary to what is expected for animals suffering focal ischaemia (Carlioni et al., 2008). One experimental stroke study using Mongolian gerbils had similar results and suggested it was due to changes in visual input (Ishibashi et al., 2003b), but mice have poorly developed visual system in comparison to gerbils (Shankar and Ellard, 2000), indicating the choice of goal arm may not be related to the visual system.

There was no difference between progesterone treated and non-treated animals in terms of survival and weight loss, which is not surprising as the aim was to cause measureable functional deficit without excessive mortality or weight loss. Also, no difference in oedema was found after all functional tests were completed, most likely due to animals having fully recovered from oedema. Progesterone treatment was found not to be beneficial in reducing neurological deficit (both 28 point and modified 29 point neurological deficit scores). Foot-fault showed progesterone to have lower contralateral foot-slips but was not significant due to lack of power. However, the modified foot-fault did find progesterone to significantly reduce foot slips, due to including animals that died in analysis. T-maze found no difference in alternation rate between progesterone treated and non-treated animals. Interestingly, progesterone treated animals did have a preference to turn left (contralaterally to ischaemic damage). Animals with ischaemic brain injury have a tendency to turn ipsilaterally, towards the damaged side during the T-maze and tests of behavioural asymmetry (Carlioni et al., 2008; Zhang et al., 2002). Progesterone has been shown to alter cognitive performance (Frye and Walf, 2008). However, mice were dosed on the day of occlusion and so progesterone would have been metabolised by the time of testing. Therefore, it is unknown why

progesterone dosed animals have a preference for the left goal arm rather than only approximately 50%, which is expected for normal behaviour. Progesterone dosed animals did take quicker to complete T-maze tasks, indicating a better functional outcome. There was no difference in oedema, most like due to the reasons given previously for the initial experiments of BPH/2. Care must be taken with these findings, as our comparison group did not receive vehicle, due to the limited number of animals available for testing. Progesterone was dissolved in DMSO, which has been shown to have neuroprotective properties (Jacob and de la Torre, 2009). Hence, it cannot be confirmed on the level neuroprotection is attributed to progesterone or DMSO and so further investigation is required to determine this.

Overall, progesterone was found to be beneficial and concurs with the findings of Kumon *et al*, who found progesterone to be neuroprotective in spontaneous hypertensive rats (Kumon et al., 2000). However, due to the low number of animals, less than ideal vehicle group and lack of shams, this needs to be viewed with caution. As a model, BPH/2 mice represent another model of hypertension in stroke research. Spontaneously hypertensive rats and the related stroke prone hypertensive rats are the most widely used hypertensive animals used in stroke research, a choice that has been criticised due to the animals limited representation of processes involved in hypertension present in humans (Pinto et al., 1998). BPH/2 mice represent another possible model of hypertension, one that is rennin-independent, while spontaneous hypertensive rats are rennin-dependent and so offers another mechanism, which can be investigated.

In conclusion, progesterone given as a loading dose (bolus i.p.) followed by maintenance (osmotic mini-pump) delivery is effective in conferring neuroprotection in a transient MCAO model in adult male mice. Aged

animals have an increased sensitivity to MCAO and did not display, in the outcomes measured here, any benefit from progesterone treatment. NOD/ShiLtJ mice are not recommended as a model of diabetes for experimental stroke research, because of their severe symptoms would result in high mortality after surgery. Hypertensive BPH/2 mice are a potential hypertensive model and had better functional outcomes after treatment with intraperitoneally administered progesterone, compared to non-treated hypertensive animals.

CHAPTER 5

Progesterone treatment for experimental stroke: an individual animal meta-analysis.

Publications contributing to this chapter:

Wong, R., Renton, C., Gibson, C.G., Murphy, S.J., Kendall, D.A., and Bath, P.M.W. (2012). Progesterone treatment for experimental stroke: an individual animal meta-analysis. *J Cereb Blood Flow Metab* 33, 1362-72 (Published)

5.1 INTRODUCTION

Studies assessing the safety and efficacy of progesterone in pre-clinical models of stroke (Alkayed et al., 2000; Chen and Chopp, 1999; Chheng-Orn Evans et al., 2004; Gibson et al., 2005b; Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002; Murphy et al., 2000; Roof et al., 1996) have previously been integrated in a systematic review and meta-analysis based on published summary ('group') data (Gibson et al., 2007). However, this systematic review suggested that key questions remained to be answered, including demonstrating efficacy as a function of sex and age and evaluating the dose and timing of progesterone administration. The gold-standard for assessing effects across studies within and between subgroups is to use a meta-analysis based on individual animal data (IAD), as previously performed for NXY-059, another putative neuroprotectant (Bath et al., 2009). The present study reports the results of a systematic review and meta-analysis based on IAD of pre-clinical studies of progesterone in experimental models of stroke.

5.2 METHODS

5.2.1 Ethics

No ethics approval was needed for the present study which follows a protocol defined at the beginning of the project based on a previous study using IAD from experimental stroke studies of NXY-059 (Bath et al., 2009).

5.2.2 Search strategy

Completed studies that investigated the effect of progesterone in animal models of focal stroke were sought via searches of electronic databases (last search: 9 October 2012) including 'Web of Knowledge' and 'PubMed'.

Search strategies used the following key words: progesterone, stroke, ischaemia. Reference lists from existing systematic and non-systematic reviews, and identified study publications, were also searched (Gibson et al., 2008b; Roof and Hall, 2000). Where duplicate publications were identified, information from the primary report was used. Publications could be in any language.

5.2.3 Selection of studies

Completed studies, randomised, and whether published or unpublished, were included if experimental focal stroke injury was induced in animal models and progesterone was administered. Stroke models could include transient or permanent focal cerebral ischemia, and progesterone or its metabolite allopregnanolone had to be administered exogenously. Studies had to include data on at least one outcome of interest: lesion volume, vital status and/or functional outcome. Studies were excluded if they did not report induction of focal cerebral ischemia, or administration of progesterone, or did not contain original data, or involved just global ischaemia.

5.2.4 Validity assessment

Studies were identified based on the inclusion/exclusion criteria listed above. The methodological quality of each study was assessed, using a nine point score modified from the original 8-point STAIR system (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). A point was given for published evidence (supplemented with information from the corresponding author) for each of the following criteria: presence of randomisation (pseudo randomised 0.5), monitoring of physiological parameters, assessment of dose response relationship, assessment of optimum time window, masked outcome measurement, assessment of

outcome at 1-3 days, assessment of outcomes 1-30 days, combined lesion volume and functional outcome; and for blinding of surgery (Bath et al., 2009).

5.2.5 Data abstraction

Two reviewers (RW, CG) identified studies. One author resolved disagreements by discussion and review (PB).

5.2.6 Data extraction

Corresponding authors of papers of interest were contacted to ask if they would be willing to join the pooling project collaboration and, if so, to share IAD with data fields listed in the project's protocol (Appendix).

Individual data for each animal was sought for: species, gender (male, young intact female, old female, ovariectomised young female); age (or weight as a surrogate); lesion volume (total, cortical, subcortical); and vital status (including information on timing and cause of death – surgery, culling due to poor health, spontaneous). Information on treatment was also obtained: time of treatment from occlusion time (hours before/after); loading and maintenance dose of progesterone; treatment duration; plasma progesterone concentration.

The following study design information was extracted either from the publication or via the corresponding author: experimental model – transient, permanent; randomisation - randomised, pseudo-randomised, not randomised; blinding of surgeon to treatment; blinding of outcome assessors to treatment. Studies were considered randomised if animals were numbered before commencement of the study and a randomisation code was used to allocate animals to treatment groups; if animals were

'picked at random' from a cage, then these studies were considered pseudo-randomised since this type of approach is open to bias.

5.2.7 Quantitative data synthesis

IAD were transferred to the project's coordinating centre in Nottingham by email attachment, e.g. Excel file. Study datasets were merged into a single Microsoft Excel sheet using common field names with one row per animal. Where individual data could not be obtained, summary data were obtained from the publication and entered into a second Excel sheet. Summary data were also created from IAD and added to this second spread-sheet to allow summary meta-analysis of all studies.

Lesion volume was recorded as either absolute volume or as a percentage of the intact contralateral hemisphere. In order to combine the different units, lesion volume was standardised by standard mean difference, i.e. the difference in means/standard deviation of score. Data from animals that have died before study completion is commonly excluded from meta-analyses of animal data, but to allow its inclusion, lesion volume was given a 'worst case' value. For studies in which lesion volume was collected as a percentage, a value of 100% was assigned for death; for studies in which lesion volume was collected as an absolute value, species-dependent maximal values were assigned – 400 mm³ for rats and 225 mm³ for mice. Analyses of lesion volume were performed with death imputed and excluded in order to confirm the robustness of conclusions. Taking account of the role of progesterone as an endogenous sex steroid, sex was trichotomised into male, young female (age<12 months), and old female or ovariectomised females. Progesterone dose was standardised to mg/kg.

Meta-analysis of individual animal data was carried out using random effects mixed modelling with covariate adjustment in SAS (version 9.3). Meta-analysis of summary data was carried out using random effects analysis in STATA (version 11). Random effects analysis was used in order to take account of the expected heterogeneity between studies caused by variations in study design, i.e. differences in species, stroke model, dosing, study practice.

All analyses were performed unadjusted (no covariates). Additionally, analyses using IAD were adjusted for the following covariates, chosen for their biological relevance or relationship to treatment: model (transient, permanent), age (young ≤ 12 , aged > 12 months), sex (male, young ovariectomised female, and aged female), time to treatment, loading progesterone dose, total progesterone dose (sum of loading and maintenance) and anaesthetic agent (halothane, isoflurane). Interaction tests between treatment and covariates were assessed if the covariate was significantly associated with the outcome.

Analyses were performed by the backwards elimination procedure. This procedure starts with all potential predictor variables in the regression model and, successively, variables are dropped one at a time, such that the resulting model has the lowest value of the information criteria. Variables are dropped on the basis of their contribution to the reduction of error sum squares or 'worst' included variable. This is stopped when the best model is reached according to the Akaike Information Criterion (AIC). The AIC is an alternative significance test to estimate quantities of interest and judges a model by how closely its fitted values tend to be to the true values. AIC serves the purpose of model comparison only and does not provide diagnostics about the fit of the model to the data.

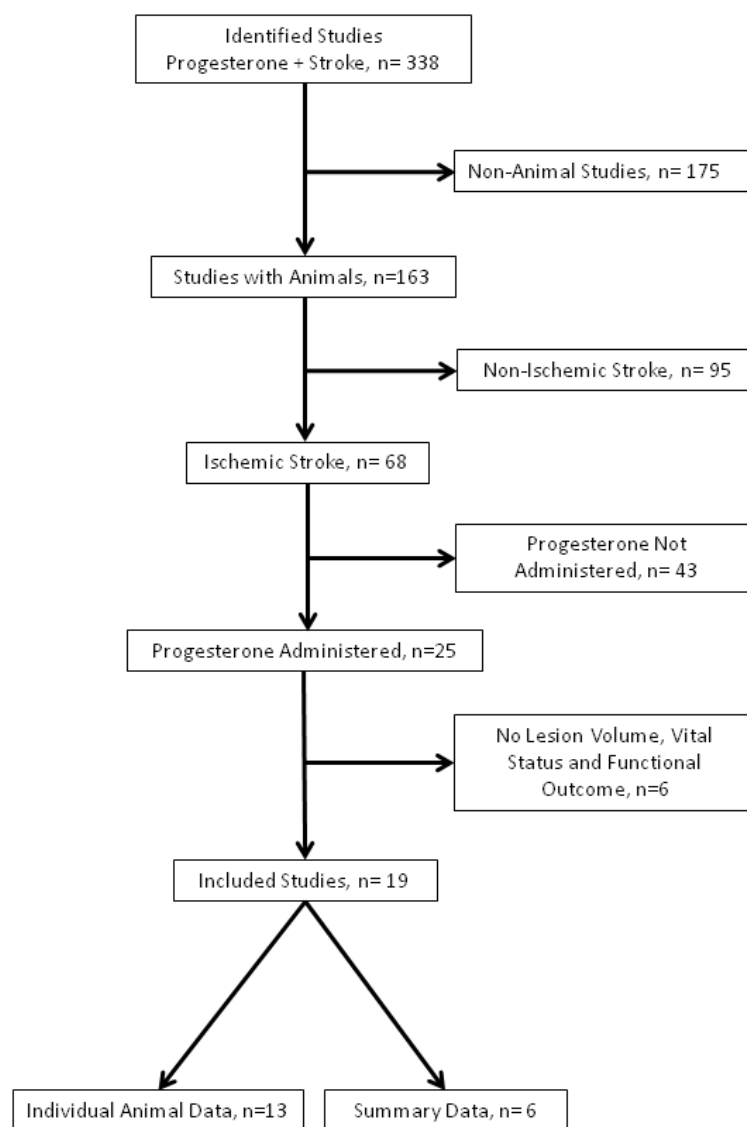
Data are given as standardised mean differences (SMD, continuous data) or odds ratios (OR, binary data) with 95% confidence intervals (95% CI), *P* value for effect, *P* value for heterogeneity, and *P* value for interaction; *P* values <0.05 are considered significant. Negative coefficients imply a reduction in lesion volume. An odds ratio less than one implies a reduction in death.

5.3 RESULTS

5.3.1 Study flow

Nineteen completed studies fulfilled the inclusion criteria (figure 5.1, table 1); since one study included two data sets (Toung et al., 2004), 20 separate datasets were used in the analyses. Authors were contacted and were asked about sharing available individual animal data for analysis. In total, IAD were received from 12 published and 2 unpublished studies. Shared data included animals excluded in publications, in particular those that died before study completion. Altogether, IAD were available for 689 animals. IAD were not shared for 6 studies in spite of repeated contact with authors (Chen and Chopp, 1999; Choi et al., 2004; Ishrat et al., 2009; Jiang et al., 1996; Kumon et al., 2000; Liu et al., 2012); nevertheless, data from these studies were included in analyses based on summary data. Five studies were excluded (table 5.3), mostly because they did not have data on total lesion volume or vital status, e.g. one study only had data on blood volume collected (Alkayed et al., 2000).

Figure 5.1: Flow chart showing study search, and included and excluded studies.



Summary data were used for studies where individual animal data were not made available. Studies were excluded if they did not report induction of focal cerebral ischaemia, administration of progesterone, or measurement of lesion volume, or did not contain original data. n = number of studies.

5.3.2 Study characteristics

Study characteristics are reported in tables 5.1 and 5.2. The studies compared the effect of exogenously administered progesterone versus no progesterone, or vehicle, on lesion volume after acute focal cerebral ischaemia. All studies used a monofilament to induce focal cerebral ischaemia in the middle cerebral artery territory. Seven studies used mice (permanent ischaemia in one, transient ischaemia in 6) and 12 studies used rats (permanent ischaemia in one, transient ischaemia in 11). The studies included 11 using young male animals, 4 using young ovariectomised females, 1 using aged males (age >12 months) and 3 using aged females. Insufficient data were available on cortical or subcortical lesion volume measurements to permit analysis and only total or hemispheric lesion volume was analysed. Dosing methods included intra-peritoneal injection (12 studies), intra-peritoneal with subcutaneous injection (4 studies) and subcutaneous implants of slow release pellets/mini-pumps (3 studies). A variety of regimens were used for progesterone administration with dosing commencing from 7-10 days before MCAO to 2 days afterwards. Lesion volume, assessed from histologically stained brain slices, was reported in mm³, % cross sectional area, or % of intact contralateral hemisphere. Study quality ranged from 2-7/9. Publication bias was apparent on visual inspection of a Begg's funnel plot based on lesion volume (figure 5.2), and when assessed statistically using Egger's test ($P=0.001$).

Table 5.1: Studies included in the analysis

Study	Lab	Species	Sex	Anaesthetic	Random	Surgery Blinded	Outcome Blinded	Temperature control	Model	Time Prog Loading Dose to Occlusion (min)	Prog Load (mg/kg)	Progesterone Maintenance (mg/kg/hr)
Responders with IAD												
Toung 2004(Toung et al., 2004)	Baltimore USA	Rat	F	Halothane (4-5% Induction, 1.25-1.5% maintenance delivery in O ₂ enriched air)	y	n	n	y	Focal (Transient)	-30	5 i.p.	5+5 i.p. for 24.5 hr
Toung 2004(Toung et al., 2004) (Prog+Est)										-30	5 i.p.	5+5 i.p. for 24.5 hr
Parker 2005(Parker S.M, 2005)	Portland USA	Mice	F	Halothane (4-5% Induction, 1.25-1.5% maintenance delivery in O ₂ enriched air)	y	n	n	y	Focal (Transient)	-7 days	none	15/30 mg s.c. 21 day release implant for 7-8 days
Murphy 2002(Murphy et al., 2002)	Baltimore USA	Rat	F	Halothane (4-5% Induction, 1.25-1.5% maintenance delivery in O ₂ enriched air)	y	n	n	y	Focal (Transient)	-30	5/10/20 i.p.	none
										-30	5 i.p.	5+5 i.p. for 24.5 hr
Murphy 2002[unpub]	Baltimore USA	Rat	F	Halothane (4-5% Induction, 1.25-1.5% maintenance delivery in O ₂ enriched air)	y	n	n	y	Focal (Transient)	-30	5 i.p.	5+5 i.p. for 24.5 hr
Murphy 2000(Murphy et al., 2000)	Baltimore USA	Rat	F	Halothane (4-5% Induction, 1.25-1.5% maintenance delivery in O ₂ enriched air)	y	n	n	y	Focal (Transient)	-30	30/60 i.p.	None
										-7 to -10 days	30/60 i.p.	30/60 i.p. daily for 7-10 days
Gibson 2004(Gibson and Murphy, 2004)	Nottingham, UK	Mice	M	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	y	y	y	Focal (Transient)	60	8 i.p.	8 + 8 i.p. for 23 or 47 hrs
Gibson 2005(Gibson et al., 2005b)	Nottingham, UK	Mice	M	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	y	y	y	Focal (Permanent)	60	8 i.p.	8 + 8 i.p. for 47 hrs
Sayed 2006(Sayed et al., 2006)	Atlanta USA	Rat	M	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	n	n	y	Focal (Transient)	115	8 i.p.	8 s.c. for 72 hrs

Intraperitoneal= I.P, Subcutaneous= S.C., F= Female, M= Male, Unpub= Unpublished, Prog= Progesterone, Time Prog Loading Dose to Occlusion (min)= Time to occlusion that the progesterone loading dose was given in minutes , Progesterone Maintenance (mg/kg/hr)= maintenance doses given (mg/kg) over time in hours from start of progesterone treatment to lesion volume measurement.

Table 5.1: Continued

Study	Lab	Species	Sex	Anaesthetic	Random	Surgery Blinded	Outcome blinded	Temperature control	Model	Time Prog loading dose to Occlusion (min)	Prog Load (mg/kg)	Progesterone Maintenance (mg/kg/hr)
Sayeed 2007(Sayeed et al., 2007)	Atlanta USA	Rat	M	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	n	y	y	Focal (Permanent)	120	8 i.p.	8 + 8 +8 s.c. for 72 hrs
Sayeed 2009(Ishrat et al., 2009)	Atlanta USA	Rat	M	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	n	n	y	Focal (Transient)	115	8 i.p.	8 s.c. for 72 hrs
Coomber 2010(Coomber and Gibson, 2010)	Leicester UK	Mice	F	Isoflurane 4% (NO2/O2 70/30% mixture)	y	y	y	y	Focal (Transient)	-7 days	none	50 mg s.c. 21 day release implant for 7 days
Gibson 2011(Gibson et al., 2011)	Leicester UK	Mice	F	Isoflurane 4% (NO ₂ /O ₂ 70/30% mixture)	y	y	y	y	Focal (Transient)	60	8 i.p.	8 + 8 i.p. for 23 or 47 hrs
Wong 2012[Unpub]	Nottingham, UK	Mice	M	Isoflurane (100% O ₂)	y	y	y	y	Focal (Transient)	30,60	8 i.p.	50mg s.c. 3 day release implant for 48 hrs
Non responders/No IAD												
Jiang 1996(Jiang et al., 1996)	Atlanta USA	Rat	M	Halothane (3.5% induction, 1-2% maintenance delivery 70% N ₂) and 30% O ₂)	y	n	n	y	Focal (Transient)	-30	4 i.p.	4 + 4 i.p. for 48 hrs
Chen 1999(Chen and Chopp, 1999)	Rochester USA	Rat	M	Halothane (3.5% induction, 1-2% maintenance delivery 70% N ₂) and 30% O ₂)	n	n	n	y	Focal (Transient)	120	4/8/32 i.p.	4 + 4/ 8 + 8/ 32 + 32 i.p. for 7 days
Ishrat 2009(Ishrat et al., 2009)	Atlanta USA	Rat	M	Isoflurane 5% induction, 1-2% maintenance, 20% NO ₂ and 20% O ₂ mix	n	n	n	y	Focal (Permanent)	60	8 i.p.	8 + 8 +8 s.c. for 72 hrs
		Rat	M	Isoflurane 5% induction, 1-2% maintenance, 20% NO ₂ and 20% O ₂ mix	n	n	n	y	Focal (Permanent)	60	8 i.p	8 + 8 + 8 s.c for 72 hrs
Kumon 2000(Kumon et al., 2000)	Ehime, Japan	Rat	M	Halothane (3% induction, 1-2% maintenance delivery 70% N20 and 30% O ₂)	y	y	y	y	Focal (Transient)	120	4 / 8 i.p.	none
Choi 2004(Choi et al., 2004)	Pusan Korea	Rat	M	Sodium thiopental (50 mg/kg)	n	n	n	y	Focal (Transient)	-24 hr	4 i.p.	4 i.p. for 48 hrs
Liu 2012(Liu et al., 2012)	Paris France	Mice	M	Ketamine (50mg/kg) and xylazine hydrochloride (6mg/kg)	y	n	y	y	Focal (Transient)	60	8 i.p.	8 + 8 i.p. for 23 or 47 hrs

Table 5.2: Experimental Results

Study	Exclusions from Analysis (Prog: Control)	Death (Prog: Control)	Lesion Volume Timing (h)	Total Lesion Animals (Prog:Control)	Total Lesion Volume (SD) (Prog: Control)	Quality/9	Comments on Progesterone
Responders with IAD							
Toung 2004(Toung et al., 2004)	5 (4:1)	5 (4:1)	24	20 (10:10)	18.61 (13.45): 24.06 (13.31)	3	Combined hormone reduced infarct volume and progesterone does not attenuate estrogen effect.
Toung 2004 (Prog+Est)(Toung et al., 2004)	2 (1:1)	2 (1:1)		20 (10:10)	7.59 (5.69): 8.92 (9.15)		
Parker 2005(Parker S.M, 2005)	0	0	23.5	24 (16:8)	49.77 (12.9): 53.46 (18.68)	4	Chronic, exogenous progesterone prior to MCAO alters ischemic brain injury in ovariectomized female mice
Murphy 2002(Murphy et al., 2002)	28 (15:13)	2	24	61 (39:22)	17.85 (10.78): 18.9 (14.37)	5	Progesterone both before MCAO and during reperfusion decreases ischemic brain injury
Murphy 2002[Unpub]	0	0	none	none	none	3	Hormone Measurement
Murphy 2000(Murphy et al., 2000)	29 (19:10)	11	24	56 (42:14)	13.32 (8.26): 8.42 (8.09)	5	Progesterone does not ameliorate histological injury after MCAO in previously ovariectomised adult female rats. Chronic progesterone administration can exacerbate infarction in subcortical regions
Gibson 2004(Gibson and Murphy, 2004)	4	4	24, 48	20 (10:10)	13.97 (5.21): 18.64 (5.9)	7	Beneficial effects of progesterone after cerebral ischemia
Gibson 2005(Gibson et al., 2005b)	6	4 (2:2)	48	24 (12:12)	82.5 (12.58): 90.87 (15.88)	5	Progesterone is neuroprotective in both permanent and transient ischemia and effect is related to suppression of the inflammatory response
Sayeed 2006(Sayeed et al., 2006)	9	2	72	13 (6:7)	15.96 (3.6): 24.38 (6.69)	4	Progesterone is effective at reducing infarct pathology
Sayeed 2007(Sayeed et al., 2007)	3	1	72	15 (7:8)	13.81 (5.68): 27.8 (6.27)	5	Progesterone is neuroprotective
Sayeed 2009(Ishrat et al., 2009)	0	0	72	16 (8:8)	9.32 (2.29): 20.12 (2.91)	4	Progesterone is neuroprotective
Coomber 2010(Coomber and Gibson, 2010)	9	4(3:1)	48	13 (6:7)	20.62 (9.22): 23.41 (10.18)	5	No effect
Gibson 2011(Gibson et al., 2011)	10	8 (5:3)	48	42 (20:22)	21.59 (7.42): 24.67 (5.68)	7	Progesterone is not beneficial in ovariectomised mice but is in Aged female mice for lesion volume
Wong 2012[Unpub]	0	0	none	none	none	3	Hormone Measurement

Unpub= Unpublished, Exclusion from Analysis: Total Number (Progesterone: Control), 192

Death: Total Number (Progesterone: Control), Total Lesion Volume (SD): Mean Raw data (Standard Deviation).

Table 5.2: Continued

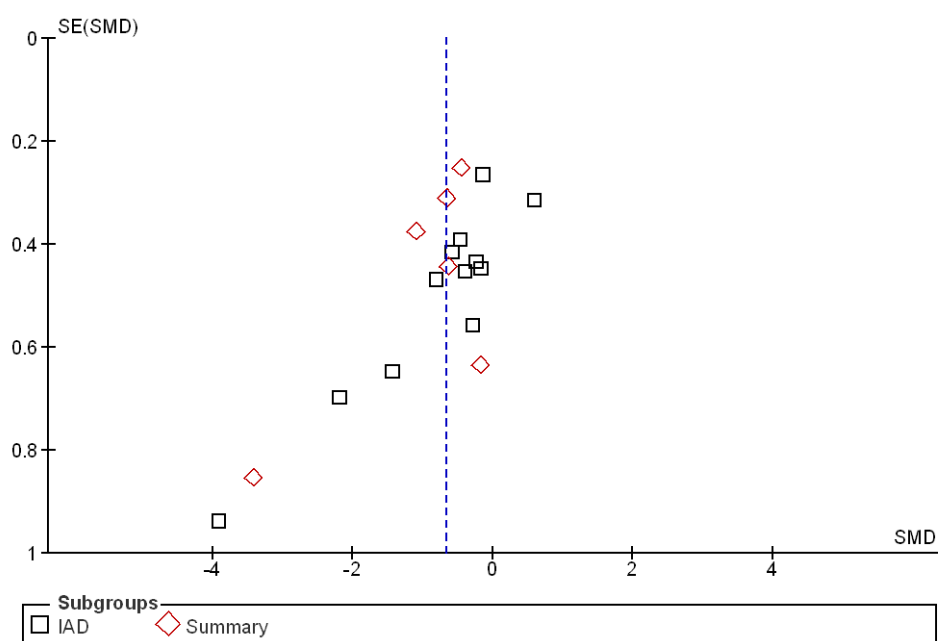
Study	Exclusions from Analysis (Prog: Control)	Death (Prog: Control)	Lesion Volume Timing (h)	Total Lesion Animals (Prog:Control)	Total Lesion Volume (Prog: Control)	Quality/9	Comments on Progesterone
Non responders/No IAD							
Jiang 1996(Jiang et al., 1996)	-	-	48	48 (24:12)	24.43 (10.39): 35.1 (15.59)	5	Progesterone administered before or after MCAO reduces ischemic cell damage and improves physiological and neurological function 2 days after stroke.
Chen 1999(Chen and Chopp, 1999)	-	-	7 days post MCAO	28 (7:7,7,7)	26.57 (11.67): 34.4 (10.5)	5	8mg/kg of progesterone reduce brain lesion and improved neurological functional deficit
Ishrat 2009(Ishrat et al., 2009)	-	-	72	24 (8:8,8)	9.24 (2.96): 20.11 (3.1)	3	Progesterone treated have reduced infarct volume and improved ability to stay on rotarod and grip test
Kumon 2000(Kumon et al., 2000)	-	-	2 or 7 days after MCAO	48 (32:16)	31.15 (14.13): 41.2 (10.4)	7	Treatment with high dose of 8mg/kg of progesterone results in reduction of lesion size, neurological deficits and body weight
Choi 2004(Choi et al., 2004)	-	-	24	11 (5:6)	210 (67.6): 231.4 (154.07)	2	No effect
Liu 2012(Liu et al., 2012)	-	-	48	64 (32:32)	43.25 (22.75): 52.25 (17.96)	5	Progesterone was neuroprotective in wild-type mice

Table 5.3: Excluded studies and reasons for exclusion after meeting inclusion criteria

Study	Reason for Exclusion
Jiang 2009 (Jiang et al., 2009)	Oedema but no total lesion volume data
Cai 2008 (Cai et al., 2008)	Number of surviving cells but no total lesion volume data
Zhang 2010 (Zhang et al., 2010)	Number of surviving cells but no total lesion volume data
Morali 2005 (Morali et al., 2005)	Number of surviving cells but no total lesion volume data
Alkayed 2000 (Alkayed et al., 2000)	Blood volume but no total lesion volume data
Dang 2011 (Dang et al., 2011)	No total lesion volume data

Ischaemic stroke induced in animals and progesterone administered.

Figure 5.2: Begg's funnel plot for studies of progesterone on lesion volume when combining individual animal and summary data.



The funnel plot relates precision (reciprocal of standard error, SE) to the standardised mean difference (SMD). Asymmetry is present indicating publication bias (Egger's test, (Egger et al., 1997) $P=0.001$).

5.3.3 Quantitative data synthesis

5.3.3.1 Lesion volume – combined individual and summary data

Due to the lack of data on individual animals that died following the start of treatment in summary data (where IAD were not shared), it was not possible to account for death in combined IAD and summary data analysis (18 studies, 512 animals).

In an unadjusted analysis, progesterone reduced standardised lesion volume (SMD -0.766, 95% CI -1.173 to -0.358, $P < 0.001$) (table 5.4, figure 5.3). In pre-defined subgroups of animals, significant reductions in lesion volume were seen with progesterone in mice, rats, male animals, male mice, male rats, and in both transient and permanent models of ischaemia. When assessed by pre-defined study design and quality markers, progesterone reduced lesion volume in studies that were randomised, had or did not have blinded surgery, had or did not have blinded outcome assessment, used isoflurane as the anaesthetic agent, and those that were of high quality (STAIR scale = 7) (table 5.4). Some sub-group analyses were of low statistical power because of limited data, e.g. sodium thiopenthal as the anaesthetic agent (1 study, 11 animals). Heterogeneity was present for some analyses confirming the need for the use of a random effects statistical model.

When assessing the effect of interactions between treatment and subgroups, progesterone had greater effects on reducing lesion volume in males than young females (figure 5.3), and when the anaesthetic was isoflurane rather than halothane (table 5.4).

Table 5.4: Effect of progesterone on standardised total lesion volume using combined individual animal and summary data, both combined and in pre-defined subgroups.

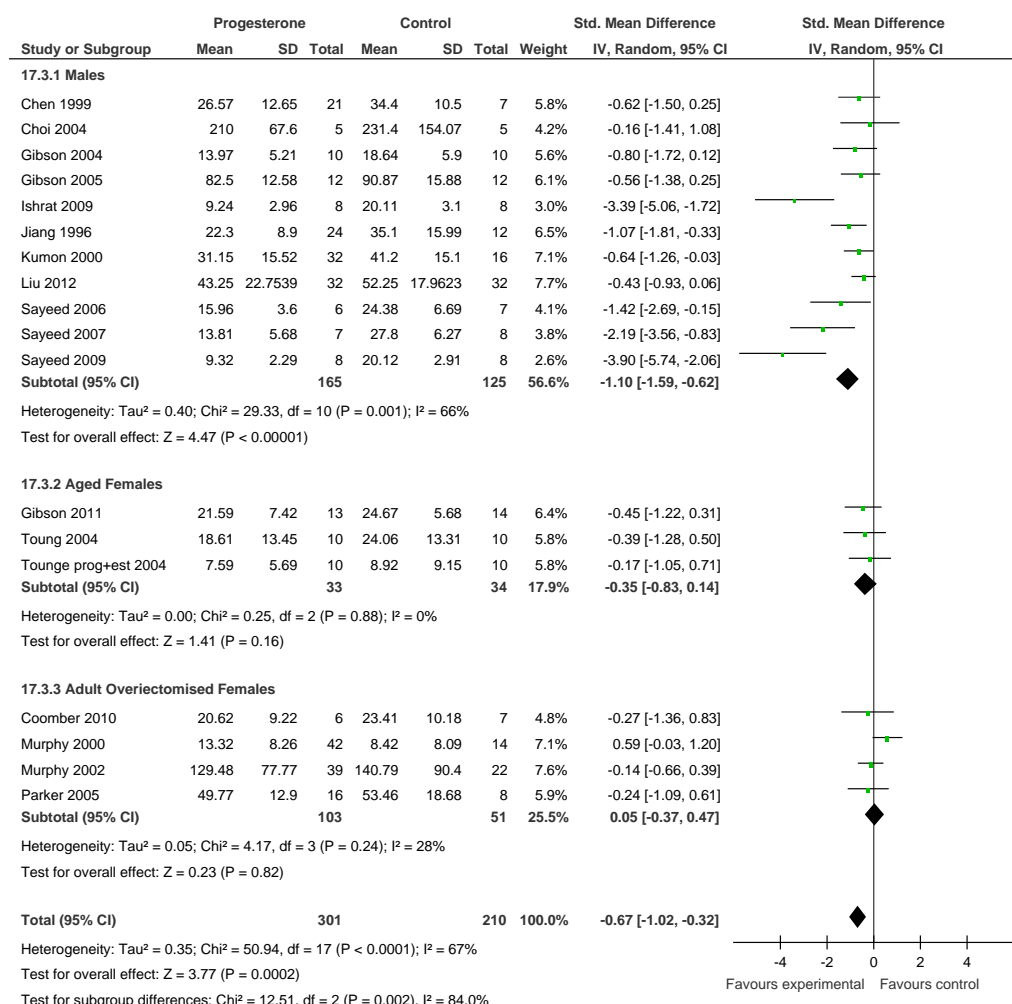
Model	Studies	Animals	SMD	95% CI	Effect P	Inter-action P
Total	17	448	-0.766	-1.173, -0.358	<0.001 †	
Species						
Mice	5	108	-0.492	-0.881, -0.102	0.013	0.44
Rats	12	340	-0.958	-1.539, -0.376	0.001 †	
Sex						
Male	10	227	-1.316	-1.902, -0.730	<0.001 †	
Female, adult	3	130	0.113	-0.437, 0.662	0.688	
Female, aged ‡	4	91	-0.333	-0.754, 0.088	0.121	
Mice						
Male	2	44	-0.697	-1.308, -0.086	0.025	
Female, adult						
Female, aged ‡						
Rats						
Male	8	183	-1.547	-2.303, -0.792	<0.001 †	
Female, adult						
Female, aged ‡						
Model						
Transient	14	393	-0.549	-0.928, -0.171	0.004 †	0.075
Permanent	3	55	-2.058	-3.850, -0.266	0.024 †	
Treatment time						
Before MCAO						
After MCAO						
Loading dose						
Maintenance dose	*					
Total dose	*					
Randomised						
Yes	14	393	-0.674	-1.088, -0.260	0.001 †	
No	3	55	-1.348	-3.046, 0.350	0.120 †	
Blinded surgery						
Yes	5	132	-0.589	-0.946, -0.233	0.001	
No	12	316	-0.934	-1.537, -0.332	0.002 †	
Blinded outcome						
Yes	6	147	-0.725	-1.134, -0.316	0.001	
No	11	301	-0.813	-1.413, -0.213	0.008 †	
Anaesthetic						
Isoflurane	8	144	-1.512	-2.324, -0.701	<0.001 †	
Halothane	8	293	-0.323	-0.700, 0.054	0.093 †	
Sodium	1	11	-0.173	-1.363, 1.016	0.775	
Thiopental						
Quality						
2	1	11	-0.173	-1.363, 1.016	0.775	
3	3	56	-1.224	-2.843, 0.395	0.138†	
4	3	53	-1.828	-3.824, 0.168	0.073†	
5	7	233	-0.534	-1.120, 0.052	0.074†	
7	3	95	-0.636	-1.061, 0.211	0.003	

† Heterogeneity present between studies

‡ or ovariectomised

Data are standardised mean differences (SMD), 95% confidence intervals (95% CI) and significance for effect and interaction. Analyses are adjusted for random effects. Result in bold are statistically significant.

Figure 5.3: Forrest plot of effect of progesterone on lesion volume using individual animal and summary data



Studies are ordered by animals' sex: male; aged females; or adult ovariectomised female.

5.3.3.2 Adjustments for analyses involving individual animal data

Using individual animal data alone, backwards elimination of covariates revealed that maintenance dose alone led to a minimum in AIC for analyses of lesion volume. For death, AIC was minimised with the combination of sex, time to treatment, loading dose, total dose and anaesthetic agent as covariates. Subsequent adjusted analyses were made using these covariates.

5.3.3.3 Lesion volume – individual animal data, death included

Clinical trials include patients who die following treatment in their outcome analyses; hence, lesion volume was imputed for animals that died following treatment. In 12 studies (337 animals), treatment with progesterone did not show any benefit on standardised lesion size, whether in an adjusted analysis (SMD -0.322, 95% CI -0.779 to +0.135, $P=0.16$) or unadjusted analysis (SMD -0.208, 95% CI -0.542 to 0.125, $P=0.20$) (table 5.5). In view of the neutral result, subgroup analyses were not performed. The unpublished studies Wong *et al* 2012 and Murphy *et al* 2002 did not contain lesion volumes and were not included in analysis.

Table 5.5: Effect of progesterone on standardised total lesion volume, including animals that died, using individual animal data overall and in pre-defined subgroups.

Model	SMD	95% CI	Effect P	Interaction P
Overall, adjusted	-0.322	-0.779, 0.135	0.16	N/A
Species, mice vs. rats	-0.033	-0.679, 0.613	0.91	N/A
Age, Aged vs. young	-0.071	-0.637, 0.495	0.80	N/A
Sex, adult female vs. male vs. aged	0.167	-0.508, 0.842	0.86	N/A
Model	-0.138	-0.705, 0.678	0.97	N/A
Time to treatment (per hour)	-0.001	-0.005, 0.004	0.72	N/A
Loading dose (per mg/kg)	-0.002	-0.020, 0.016	0.83	N/A
Total dose (per mg/kg)	0.003	-0.002, 0.001	0.18	N/A
Anaesthetic, halothane vs. isoflurane	-0.034	-0.789, 0.721	0.93	N/A

N/A: Not appropriate; N/D: Not done - no deaths in a comparator group

Animals that died were assigned a lesion volume of 100%, 1000*mm³ for rats or 225mm³ for mice. Data are standardised mean differences (SMD), 95% confidence intervals (95% CI) and significance for effect and interaction. Analyses are adjusted for random effects and species, age, sex, model, time to treatment, loading dose, total dose and anaesthetic agent. Result in bold are statistically significant.

5.3.3.4 Lesion volume – individual animal data, death excluded

Using individual animal data alone and ignoring animals that died (12 studies, 309 animals), progesterone reduced standardised lesion volume in both adjusted (SMD -0.349, 95% CI -0.672 to -0.025, $P=0.035$) and unadjusted (SMD -0.451, 95% CI -0.735 to -0.166, $P=0.0035$) analyses (table 5.6). Progesterone reduced standardised lesion volume in pre-defined subgroups: males, male rats, animals anaesthetised with isoflurane, and when outcome was measured blinded to outcome. The unpublished studies Wong *et al* 2012 and Murphy *et al* 2002 did not contain lesion volumes and were not included in analysis.

Table 5.6: Effect of progesterone on standardised total lesion volume, excluding animals that died, using individual animal data overall and in pre-defined subgroups.

Model	SMD	95% CI	Effect P	Interaction P
Overall, adjusted	- 0.585	-0.954, -0.215	0.004	N/A
Species, mice vs. rats	-0.031	-0.540, 0.478	0.90	N/A
Age, Aged vs. young	-0.037	-0.616, 0.541	0.90	N/A
Sex, adult female vs. male vs. Aged females	0.130	-0.445, 0.706	0.87	N/A
Model, permanent vs. transient	0.004	-0.535, 0.543	0.99	N/A
Time to treatment (per hour)	-0.001	-0.005, 0.003	0.58	N/A
Loading dose (per mg/kg)	0.011	-0.006, 0.028	0.20	N/A
Total dose (per mg/kg)	-0.001	-0.005, 0.004	0.80	N/A
Anaesthetic (halothane vs. isoflurane)	-0.000	-0.589, 0.588	1.00	0.80

Data are standardised mean differences, 95% confidence intervals and significance for effect and interaction. Analyses are adjusted for random effects and maintenance dose of progesterone. Result in bold are statistically significant.

5.3.3.5 Death

Using individual animal data alone (14 studies, 503 animals, 58 deaths), progesterone was associated with an increase in death, significantly so in an adjusted analysis (odds ratio 2.64, 95% CI 1.17 to 5.97, $P=0.020$), and with a trend in an unadjusted analysis (OR 1.81, 95% CI 0.70 to 4.66, $p=0.22$) (table 5.7, figure 5.4). When assessed in predefined subgroups, the increase in death rate was most prominent in mice, older animals, young ovariectomised females (versus males or old females/ovariectomised young females), and with later administration of

drug. A significant interaction was present between treatment and species (table 5.7).

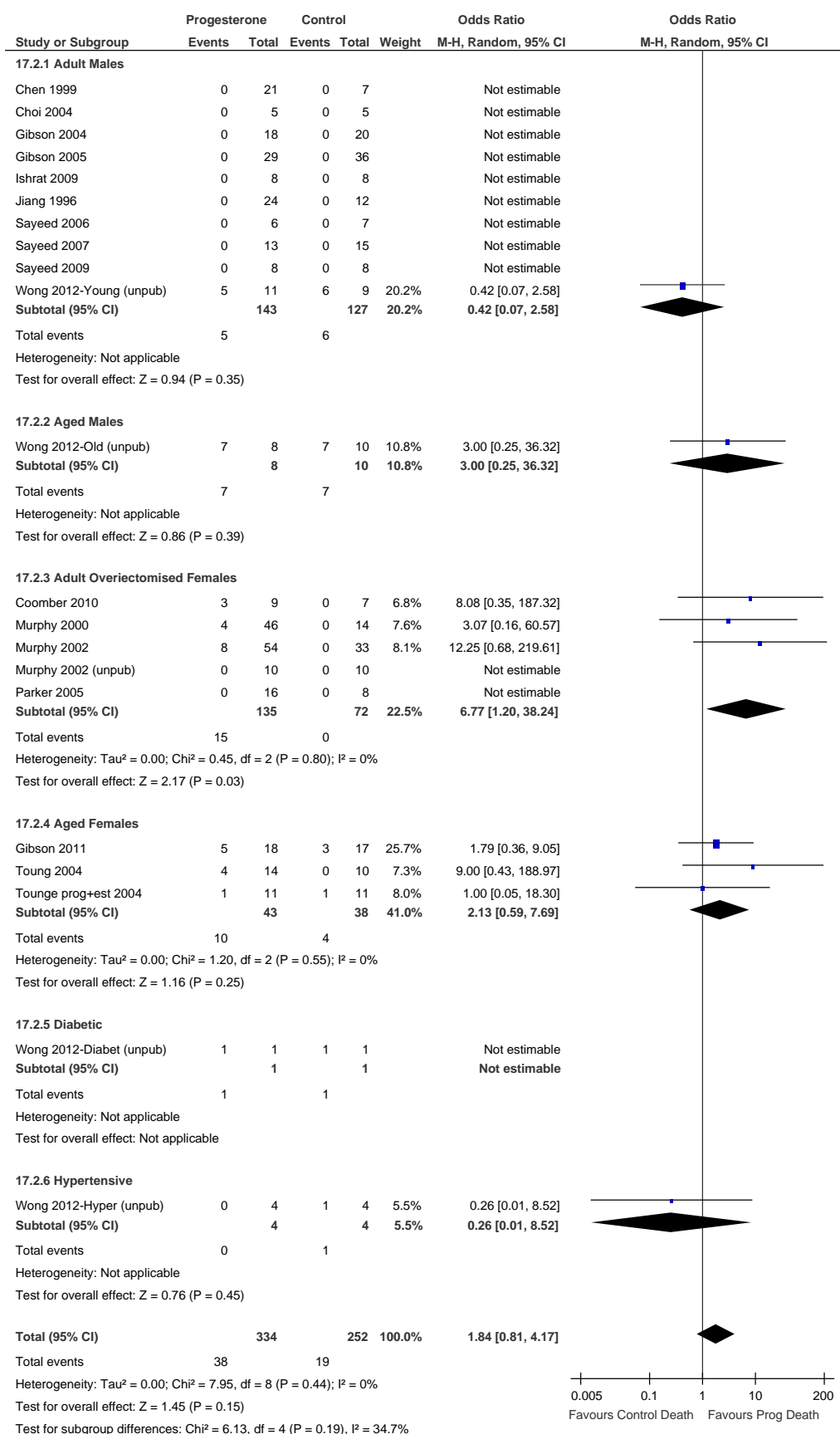
Table 5.7: Effect of progesterone on death using individual animal *and summary* data overall and in pre-defined subgroups.

Model	SMD	95% CI	Effect P	Interaction P
Overall, adjusted	2.64	1.17-5.97	0.020	N/A
Species, mice vs. rats	10.35	2.14-49.93	0.004	0.028
Age, Aged vs. young	11.70	1.70-80.20	0.012	0.76
Sex, adult female vs. male vs. aged females	11.68	1.79-76.34	0.028	N/D
Model, transient vs. permanent	-	-	NC	N/D
Time to treatment (per hour)	1.01	1.00-1.01	0.025	N/A
Loading dose (per mg/kg)	0.89	0.69-1.15	0.36	N/A
Total dose (load + maintenance, per mg/g)	1.03	0.99-1.06	0.16	N/A
Anaesthetic, halothane vs. isoflurane	1.11	0.25-4.97	0.89	N/A

N/A: Not appropriate; N/D: Not done - no deaths in a comparator group

Data are odds ratios, 95% confidence intervals, and significance for effect and interaction. Analyses are adjusted for random effects and species, age, sex, model, time to treatment, loading dose, total dose and anaesthetic agent. Subgroup analyses of sex consisted of young ovariectomised females versus males, or aged females, or aged females versus males. Young ovariectomised females were found to have significantly more deaths and these results were presented. Result in bold are statistically significant.

Figure 5.4: Forrest plot of effect of progesterone on death using individual animal and summary data.

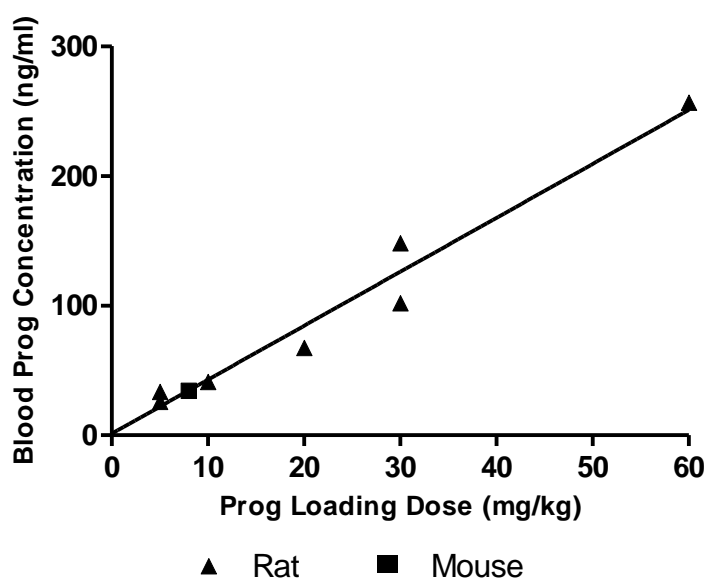


5.3.3.6 Progesterone blood concentration

The relationship between progesterone loading dose (mg/kg) and blood concentration (ng/ml) was assessed in four studies on rats (Murphy *et al* 2000, 2002, Parker *et al* 2005 and Murphy 2002 unpublished (Murphy *et al.*, 2002; Murphy *et al.*, 2000; Parker S.M, 2005)) and one in mice (Wong *et al* 2012 (Wong *et al.*, 2012)). Some studies did not involve stroke models (Murphy 2002 unpublished and Wong *et al* 2012 (Wong *et al.*, 2012)). Blood progesterone concentration was measured 1 hour from the start of treatment. A linear concentration-dose relationship was seen ($P<0.0001$, figure 5.5) with:

$$[\text{Progesterone}] = 1.304 + 4.156 \times \text{progesterone loading dose}$$

Figure 5.5: Relationship between blood concentration and loading dose of progesterone.



Progesterone blood concentration was measured at 1 hour from the start of treatment. Equation of best fit [Progesterone] in blood (ng/ml) = $4.156 \times \text{progesterone loading dose (mg/kg)} + 1.304$.

5.4 DISCUSSION

In our earlier systematic review and meta-analysis, based on published summary data, progesterone was associated, in a dose-dependent manner, with reduced lesion volume following experimental brain injury (Gibson et al., 2008b). However, meta-analysis based on summary data does not allow subgroup analyses to be performed, unlike a meta-analysis based on IAD which does. Therefore, it was not possible to assess the effect of treatment on death or in sub-groups of animals or experimental designs. In the present systematic review and meta-analysis, data for individual animals, including those that died, was assessed from both published and unpublished studies focusing on progesterone in surgically-induced stroke. Such meta-analyses are considered to be the gold standard for assessment of intervention effects (Stewart and Parmar, 1993). The main findings were that progesterone reduced lesion volume but increased the incidence of stroke-related death. In a pharmacokinetic sub-study, progesterone concentration in the blood was proportional to loading dose (figure 5.5).

Progesterone was found to reduce lesion volume in analyses based on both combined IAD and summary data (which excluded animals that died post-treatment), or using IAD alone where animals that died were excluded. Although analyses based on summary data or IAD varied somewhat (fewer significant effects in the IAD analysis compared to summary data, most likely due to summary data having more statistical power from having a greater number of animals), lesion volume was reduced regardless of rodent species (mice and rats) or model of occlusion (transient and permanent) yet only in males. However, when animals that died were included in the analysis of lesion volume (amounting to analysis of the

composite outcome of lesion volume or death), progesterone exerted no beneficial effect. The explanation is that progesterone increased the incidence of death, particularly in mice versus rats, older animals versus younger, young ovariectomised females versus males or older females, and when administered late in the protocol.

Several drug-subgroup interactions bear further comment. The administration of exogenous progesterone appeared to be detrimental in young ovariectomised females as compared to males (as seen previously (Gibson et al., 2008b)) and older females, indicating other factors involved, other than endogenous levels of progesterone. This finding has been observed previously in individual studies; e.g. Gibson & Murphy (2004), who found progesterone to aid survival after ischaemia in young male mice (Gibson and Murphy, 2004), although others found no effect (Alkayed et al., 2000; Coomber and Gibson, 2010; Gibson et al., 2011). Other studies have either not investigated survival or the reported numbers of deaths are small, thereby preventing detailed analysis. Importantly, only death post-treatment should be included, and earlier culling, e.g. due to inadequate occlusion (as determined using laser Doppler), does not amount to attrition bias. Equally, animals that have to be killed humanely after treatment because they are in poor condition, e.g. they exhibit barrel-rolling, must be included in the numbers that died. The application of humane endpoints to inform decisions regarding termination of animals will vary between institutions and so it is important to include these animals in this type of analysis. A large proportion of studies had more deaths from progesterone treatment versus control (figure 5.4), and even when adjusted for sex progesterone is still associated with increased death (table 5.7), indicating other factors independent of sex could be involved.

The finding that progesterone appears to reduce lesion size but increase death is important since most pre-clinical studies only report the former. This may not be surprising since such studies are usually small and, therefore, do not have sufficient statistical power to individually assess death. However, the issue of low numbers of deaths also applies to clinical trials, and they typically report both death alone, and the combination of death and poor non-fatal outcome (lesion size in this study). The problem is that published studies don't report death and that actually this effect on death has been revealed through analysis of IAD by contacting authors. Several potential neuroprotectants have been reported to be protective in pre-clinical studies but hazardous in clinical trials, these including DCLHb, enlimomab, selfotel and tirilazad (2000; Davis et al., 2000; Furuya et al., 2001; Saxena et al., 1999). It is interesting to speculate on whether these agents increased death in pre-clinical studies, and whether clinical trials would have proceeded if IAD meta-analyses had been performed beforehand. Pre-clinical studies and analysis should strive to reflect the design of clinical trials to screen out ineffective and even deleterious interventions before reaching human testing. Nevertheless, IAD meta-analyses of pre-clinical studies may not successfully predict the results of clinical trials. In the only other IAD meta-analysis performed to date, NXY-059 was found to reduce stroke lesion volume but it failed to be effective in clinical trial (Bath et al., 2009; Diener et al., 2008; Shuaib et al., 2007a). Our results, and previous experience in experimental stroke and clinical trials, suggest that it is essential that pre-clinical studies are analysed rigorously, including performing IAD meta-analysis, before clinical trials are initiated.

Several caveats concerning the present study need to be considered; some reflecting issues with the meta-analysis itself and others concerned with

the relative paucity of experimental data relating to progesterone. First, there was evidence of significant publication bias on the basis of the effects of progesterone on lesion volume when assessed using summary data assessed both visually (figure 5.2) and using Egger's test (Egger and Davey Smith, 1998). Missing studies may well have been neutral, or even negative, raising the possibility that progesterone does not, in fact, have neuroprotective effects in cerebral ischaemia.

Second, although 18 studies (19 datasets) were identified as being potentially relevant, IAD were only obtained from 12 of these. Data from 6 trials could not be obtained in spite of repeated requests to the authors. In the absence of any response from the authors it is difficult to gauge whether these studies were unusual in any respect. However, only one of these studies had extremely positive data for lesion volume (figure 5.2) so it is unlikely that the overall results for lesion volume would have changed significantly if IAD had been available for all studies.

Third, data were only available for rodents (mice and rats), so effects in primates or any other "second species" could not be assessed (in contrast to NXY-059 for which marmosets were also studied (Bath et al., 2009). Differences in stroke outcomes and the effect of potential neuroprotectants on them, between agyric and gyric brains have been well discussed previously. Rodents have agyric brains and so possess a higher proportion of white matter compared to grey and so do not represent cortical ratios found in humans (Mehra et al., 2012). The STAIR recommendations suggests a sequential hierarchy of drug and device testing from small animal models to large animals stroke models prior to initiating enrolment for clinical trials (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). Fourth, most data involved young male animals (n=11) and few

related to aged males, or females. Further experiments in older animals are required before the translational potential of progesterone treatment can be predicted. Nevertheless, the data suggest that progesterone may be hazardous in young females.

Fifth, few animals had any co-morbidities such as hypertension or diabetes, as are commonly present in human stroke victims (Ankolekar et al., 2012). The presence of co-morbidities, such as hypertension, might attenuate neuroprotective effects, as seen with NXY-059 (Bath et al., 2009).

Sixth, lesion volume data were only available for total lesion size and not for cortical or subcortical damage. Many putative neuroprotectants appear to exert most of their effect on cortical stroke (Bailey et al., 2009b), which is a potential disadvantage, since many human strokes only involve small subcortical (lacunar) lesions.

Seventh, studies should assess functional measures as well as lesion volume and few of the included studies provided such data. This contrasts with our previous IAD meta-analysis of NXY-059 in which data were also available on motor function (Bath et al., 2009). Ideally functional measures should include death, as does the modified Rankin Scale, the preferred outcome in acute stroke trials (Lees et al., 2012; in press).

Eighth, although both transient and permanent models of stroke were represented, all experimental protocols involved stroke induction with a monofilament. In general, it is better if experiments utilise a number of different systems for inducing ischaemia. Each different system will have their strengths and weaknesses e.g. cerebral vessel occlusion with a

monofilament is highly reproducible and is reversible but do not accurately replicate the hemodynamic features of thrombolytic reperfusion (Mehra et al., 2012).

Ninth, although a wide range of time between stroke induction to initiation of treatment (ranging from 7-10 days before MCAO to 2 hrs afterwards), only post-stroke treatment is relevant to human stroke, so that many of the experiments do not really contribute data relevant to the decision on whether it is appropriate to take this treatment into man.

Tenth, it has been suggested that anaesthetics without their own neuroprotective activity should be used to avoid confounding the effects of the potential neuroprotectant under examination with those of the anaesthetic agent. However, isoflurane, an aesthetic with well described neuroprotective potential (Sanders et al., 2005) was used in most experiments, and was associated with a lower lesion volume than in experiments using halothane. However, halothane use is in decline due to its potential hepatotoxic effects and the increased availability of other inhalational agents with fewer systemic side effects. (Kitano et al., 2007)

Finally, the studies were of varying quality with respect to randomisation and blinding of surgery and outcome assessment, so that selection, performance and observer bias may have been present. However, where adjusted, analysis of lesion volume appeared to be related to high rather than low study quality, as found in our earlier summary-based meta-analysis of progesterone (Gibson et al., 2008b). This contrasts with the common finding in meta-analyses of pre-clinical studies of neuroprotection being reported in low quality studies (Crossley et al., 2008). Other quality

markers were missing in many of the included studies, including presence of a sample size calculation.

In conclusion, whilst progesterone might reduce ischaemic lesion volume, it also appears to increase the incidence of stroke-related death. Its negative effects appear to be particularly evident in young ovariectomised female animals, highlighting the fact that endogenous hormone background needs to be taken into account in experimental stroke studies. These findings suggest that clinical trials, for any potential neuroprotectant, should not be commenced until an IAD-based meta-analysis of pre-clinical data has been performed. Publications and meta-analysis should include death, and combined death and lesion volume as outcomes, since interventions may have both positive and negative effects. To enable such analysis, authors of pre-clinical studies should be encouraged to share their data with IAD pooling projects as is common practice in clinical medicine.

CHAPTER 6

General Discussion

6.1 GENERAL DISCUSSION

There are still gaps in our understanding of the neuroprotective properties of progesterone and the purpose of this project was to clarify some of these issues. The aims of the studies reported herein were to investigate; the pharmacokinetics of progesterone, the efficacy of progesterone administration via osmotic mini-pump in conferring neuroprotection after the onset of experimental stroke and the influence of co-morbidities on the effectiveness of neuroprotection from progesterone. In addition to investigating *in vivo* aspects, progesterone actions was explored *in vitro* by studying the effects of progesterone on NO production and microglia viability, as well as the viability of SHSY5Y neuroblastoma cells, subjected to simple models of OGD that are associated with ischaemia. Finally, to better understand the overall neuroprotective effectiveness of progesterone, by means of a systematic review and meta-analysis, based on IAD of pre-clinical studies of progesterone in experimental models of stroke.

Previous studies demonstrating the neuroprotective properties of progesterone, following experimental stroke in animals, have used a dosing regimen of 8mg/kg progesterone injected intraperitoneally (Gibson et al., 2005b; Gibson et al., 2011; Gibson and Murphy, 2004; Ishrat et al., 2009; Kumon et al., 2000; Sayeed et al., 2009; Sayeed et al., 2007). However, the progesterone pharmacokinetic profile in both plasma and brain, following intraperitoneal administration was not established. Progesterone delivered via a single i.p. injection had a very short half-life in both plasma and brain. Despite delivering only transient peaks of the steroid in the brain, this dosing method was successful in providing neuroprotection to young male mice (Gibson and Murphy, 2004). Progesterone treatment seems to be most effective 0-2hrs following

cerebral ischaemia (Gibson et al., 2007), although no studies in pre-clinical stroke have administered progesterone after this period. Therefore, an early, high transient peak of progesterone may be enough to initiate neuroprotection. Although, the ideal profile would be to maintain progesterone concentration at as high as possible, due to the lack of toxicity from the steroid (Gaver et al., 1985; Little et al., 1974) and continuous drug administration has the advantage of avoiding the associated oscillating drug concentrations, produced by intermittent bolus administration. Continuous progesterone administration has been shown to produce enhanced benefit in traumatic brain injury (TBI) as compared to repeated injections in a rat model (Cutler et al., 2006a). This may also be the case with progesterone in stroke and a continuous dosing method would allow tapering off progesterone to be more easily achieved.

Infusion methods are commonly used to maintain drug concentrations within the therapeutic range. Delivering progesterone via osmotic mini-pumps has not been employed prior to this study and so information about the release characteristics was investigated. It was hypothesised that osmotic mini-pump infusion could be more effective in delivering progesterone to the target organ of the brain, when compared to a bolus intraperitoneal injection. Osmotic mini-pump delivery (in conjunction with a bolus i.p loading dose) was found to be an effective way of delivering high, maintained concentrations of progesterone to the target areas of the brain (higher than the peak of pregnancy), which supports our hypothesis. Also, progesterone appears to distribute itself equally in areas of predominant white and gray matter, which is encouraging as the lack of white matter protection may be a contributing factor in the failure of experimental neuroprotective strategies (Cheng et al., 2004; Ho et al., 2005). However, caution is needed when extrapolating results from rodent models to

humans as the amount of white matter in rodents is far smaller in comparison (Green, 2003). Also, it was hypothesised that animals receiving progesterone, via osmotic mini-pump infusion, will have a better outcome compared to vehicle treatment. Osmotic mini-pump delivery (in conjunction with a bolus i.p loading dose) of progesterone following the onset of transient MCAO was found to be beneficial in terms of promoting functional recovery in the current investigation, supporting the hypothesis.

The pharmacokinetics of progesterone showing the steroid's ability to enter the brain quickly and to be maintained in high concentrations are desirable traits for a clinical neuroprotectant. The time immediately after the onset of stroke is the most crucial in terms of neuroprotectant efficacy (Ginsberg, 2008). As well as factoring in the amount of time taken for a neuroprotectant to enter the brain after it has been administered, the timing of administration also needs to be considered. The ASSIST study showed that, in the UK, only around 37% of patients with suspected stroke reached hospital within 3hrs from the onset of symptoms and only 50% within 6hrs of the onset of symptoms (Harraf et al., 2002). Therefore, experimental studies must consider the efficacy of neuroprotectants beyond a few hours after stroke onset. In the meta-analysis presented in Chapter 5, the longest delay from the initiation of experimental stroke to treatment in the studies identified was 2 hrs in rats and 1hr in mice. This was also found for the systematic review of progesterone for the treatment of experimental brain injury (Gibson et al., 2008a). Further investigation is required to explore the effectiveness of progesterone beyond 2hrs after onset of experimental stroke. Also, future clinical trials may wish to consider treatment by paramedics in the ambulance in order to administer potential neuroprotectants such as progesterone more quickly. Pilot clinical trials for the administration of drugs for stroke therapy by

paramedics are feasible and have been conducted in the past (Saver et al., 2004; Shaw et al., 2011). Already, clinical trials investigating the neuroprotective properties of progesterone following TBI have shown the hormone to be beneficial and safe (Wright et al., 2007; Xiao et al., 2008). In these trials, steady-state serum concentrations of progesterone were achieved, following continuous intravenous infusion in patients with acute moderate to severe TBI injury (Wright et al., 2005). Progesterone efficacy in stroke therapy is feasible due to the similar pathological mechanisms involved in both TBI and stroke.

There is considerable evidence to suggest that microglial activation precedes and predominates over macrophage infiltration into the brain after cerebral ischaemia (Jin et al., 2010). Active microglia, are particularly detrimental for the injured brain and contribute to infarct volume (Schilling et al., 2003; Schilling et al., 2005). The iNOS gene is transcriptionally activated in active microglia, resulting in the production of NO and substantial evidence supports the involvement of NO in ischaemic brain injury (Moro et al., 2004). Progesterone has been shown to suppress the inflammatory response and iNOS expression following cerebral ischaemia *in vivo* (Gibson et al., 2005b). Therefore, we hypothesised progesterone to attenuate NO production in microglia. Also, glutamate overload is a key neurotoxic effect that is thought to occur following brain injury, including acute ischaemic stroke. The molecular mechanisms of glutamate-induced excitotoxicity have been investigated with *in vitro* neuronal models, and progesterone has been shown to protect against excitotoxicity in dissociated cell cultures (Luoma et al., 2011; Mannella et al., 2009; Nilsen and Brinton, 2003; Ogata et al., 1993). Ischaemia models have been used to reduce viability in neuronal cultures and progesterone was shown to provide protection in a concentration-dependent manner via GABA_A

receptor activation, thereby reducing excitotoxicity in purkinje neurons (Ardeshiri et al., 2006). These studies have lead to our hypothesis that progesterone will reduce cell death.

Neither of the microglial cell lines, BV-2 and HAPI cells used in the present studies produced elevations in NO formation under ischaemic conditions. Production of NO from primary microglia and microglial cell lines (including BV-2) in hypoxic conditions without any pre-activation has been demonstrated in other studies (Lu et al., 2006; Son et al., 2009). Microglia may require activation conditions, such as exposure to LPS or cytokines including TNF- α and IFN γ in order to produce NO. Conventionally, it has been thought that, microglia cells are activated by signals originating from dying neurons (Gehrmann et al., 1995; Gonzalez-Scarano and Baltuch, 1999), and further experiments are required, in order to determine the effects of pre-activation with cytokines TNF- α and IFN γ , ATP etc in ischaemic conditions. Also, co-cultures with neurones could clarify the interactions involved between microglia and neurones challenged with ischaemia.

The *in vitro* model of ischaemia, OGD, reduced viability of both microglial and neuronal cells and progesterone *increased* cell death, compared to non-treated cells in these conditions, which conflicts against our hypothesis. This was unexpected, as *in vivo* studies have found progesterone to be neuroprotective (Gibson et al., 2005b; Gibson and Murphy, 2004) However, Murphy et al (2000) did find daily administration of progesterone for a week prior to ischaemia exacerbated brain injury in ovariectomised rats. The authors speculated that the detrimental effects was due to modulation of the GABA system by a sharp decline of progesterone after the pre-stroke treatment (Murphy et al., 2000). This is unlikely to be the case in this investigation, as cells were continuously

treated throughout OGD and re-oxygenation. Necrosis from ischaemia is characterised by cellular metabolism failure and loss of membrane integrity (Padosch et al., 2001). Progesterone could possibly aggravate this further, as steroid hormones, particular progesterone, can readily insert into bilayers and perturb plasma membrane functions (Snart and Wilson, 1967), resulting in increased cell death.

A possibility for why neuroprotection was not observed, in the presence of progesterone in the present study may be due to the cell types employed. Coughlan *et al* found progesterone to protect PC12 neurons deprived of trophic support but not cerebellar granule neurons (Coughlan et al., 2009). It is possible that cells from different brain regions or different cell lines may require a combination of sex steroids present, in order to benefit from neuroprotection. Some neurones have been observed to respond in a sexually dimorphic manner, while other neurones in different brain regions do not. Other studies found cellular stress responses, leading to cell death to be gender-dependent, (Du et al., 2004; Lieb et al., 1995), while others found no such responses to ischaemic insult in other cell lines (Ardeshiri et al., 2006). Cellular responses to ischaemia can vary due to gender and so responses to therapy could be affected in a similar manner.

Progesterone's ability to reduce NO has been demonstrated *in vitro* previously in microglia. Also, the present investigation identified, that PR did not have a major role in progesterone's attenuation of NO production in a microglial cell line. Although, the involvement of PR in the mechanism of neuroprotection by progesterone has been identified in an *in vivo* study of PR knock-out mice (Liu et al., 2012). However, further investigation is required on primary cells and *in vivo* to determine the importance of progesterone, PR and NO formation by microglia in stroke.

Previous studies have utilised LPS to investigate the inflammatory response of microglia without the use of hypoxia (Horvath et al., 2008; Jiang et al., 2011). Therefore, LPS was used to stimulate NO production in order to investigate the effects of progesterone. Progesterone reduced NO production after challenging microglia with LPS and supports our hypothesis. However, blocking the PR receptor did not inhibit the attenuating effect of progesterone on NO production, indicating other mechanisms not involving the PR mediated pathway. In a previous study Drew *et al* (2000), also found progesterone to attenuate LPS-stimulated NO production (Drew and Chavis, 2000), although, unlike in the present study, the authors did not investigate the use of a PR receptor antagonist to investigate the mechanism of the effect. Oestrogen-mediated attenuation of microglial NO production has also been demonstrated. A possible non-PR mediated mechanism for NO reduction from progesterone is iNOS inhibition through binding to the glucocorticoid response element in murine macrophages (Miller et al., 1996). However, Kohmura *et al* (2000) found progesterone mainly uses a receptor other than the glucocorticoid receptor (Kohmura et al., 2000), but at a high enough concentrations, it is possibly for progesterone to use the glucocorticoid receptor (Werb et al., 1978).

OGD *in vitro* modelling is relatively simple to perform and has the advantage of allowing key mechanisms to be identified and studied, without counter regulations that are found in complex systems being present. Although, neuroprotectants are, of course, required to go through *in vivo* stages of testing, due to the complexities of biological systems.

The sustained delivery of progesterone in young C57 Bl/6 mice, initiated following the onset of transient MCAO, was beneficial in terms of promoting functional recovery. In the present study, our outcomes have focused on

functional measures, as clinical trials of novel stroke therapies use functional measures as their primary end-point. In humans, the size of the lesion from stroke does not always correlate well with functional impairment (Pineiro et al., 2000). It is important for pre-clinical studies to have functional measures relevant to stroke outcomes in patients, as the major goal for the treatment of stroke is for patients to be functionally independent and, therefore, pre-clinical studies need to reflect the importance of functional outcomes. Restoration of behavioural function needs to be demonstrated in the pre-clinical evaluation of any putative stroke therapy. However, pre-clinical studies vary in terms of species, age, sex and doses, all of which might be contributing factors to variations in progesterone's apparent efficacy as a neuroprotectant.

In the present investigation, functional outcomes have also taken into account animals that died, by giving them a higher score than the maximum in analysis. However, care must be taken when considering death as a factor in analysis, as the value of death can be arbitrary and a high-death rate can significantly influence the overall outcome. Although, giving animals that died, a higher score in analysis can be useful in determining whether the benefits of neuroprotection, outweigh the potential increase of deaths, due to adverse effects.

In humans, age is a predictor of worse outcome following stroke. This investigation has also shown this to be true in aged animals. Aged animals were very susceptible to MCAO, even when occlusion time was greatly reduced and the aged mice did not display, in the outcomes measured here, any benefit from progesterone treatment. Animals used in the present study were in the age range of 15-16 months, which is a period considered to be the equivalent of human middle to old age. Middle age, in both mice and rats, is considered to be 10-15 months and old age is

considered to be 18-24 months. In the upper range of 14-15 months of middle age, most biomarkers may not have changed to their full extent and some have yet to start changing (Flurkey K, 2007). A limited number of studies have investigated neuroprotection of progesterone in this age range in rodents, but mostly in animals treated prior to occlusion. Toungh *et al* found female rats, 14-18 months of age, did not benefit from pre-MCAO treatment with progesterone (Toungh *et al.*, 2004), while Alkayed *et al* using 16 month old female rats found progesterone to be beneficial when administered prior to MCAO (Alkayed *et al.*, 2000). Studies have not been previously performed in aged mice that are as old as the ones investigated in the present study. Although, Gibson *et al* did use 12 months old female mice that were pre-treated with progesterone before occlusion and this was found to be protective (Gibson *et al.*, 2011). Presently only one study has administered progesterone, post-onset of occlusion in old animals; in this, Wang *et al* found progesterone to improve outcomes in 24 month old male rats (Wang *et al.*, 2010). Overall, studies of progesterone and experimental stroke involving aged animals are limited, particularly for the treatment of animals after the start of occlusion. The present study did not consider gender as a factor in progesterone's efficacy and in the future, it would be useful to address this, since post-menopausal females represent the population at highest risk of stroke (Appelros *et al.*, 2009).

On the basis of the present study, NOD/ShiLtJ mice would not be recommended, as a model of diabetes for experimental stroke research because of their severe symptoms, which we found to contribute to a high mortality rate after surgery. The surviving animals were monitored for the severity of diabetic symptoms and only a few animals were found to have high glucose in their urine and considered to be symptomatic of diabetes. High ketones in blood, an indicator of type-1 diabetes at the end of

monitoring, helped to confirm this. Monitoring of symptomatic mice found reduced weight compared to non-symptomatic mice, which was not surprising, as NOD mice are a model of spontaneous type one diabetes and is known to be susceptible to weight loss at the onset, similar to patients that develop the condition (Kikutani and Makino, 1992). Also, symptomatic NOD mice had increased thirst, which is characteristic for the onset of symptoms (Zhao et al., 2008). Although, this strain has characteristics of type-1 diabetes, the development of diabetes in these animals can be abrupt and age-dependent, and this can vary between animals. Therefore, constant monitoring is required and the number of animals that can undergo surgery at any one time is limited. Also, the excessive thirst found in this investigation for symptomatic mice makes them unlikely to survive post-surgery due to their demand for water intake.

Studies have used other diabetic rodent strains to investigate experimental stroke, including type-1 (Toung et al., 2000) and type-2 diabetic strains (Tureyen et al., 2011; Vannucci et al., 2001). There are no studies utilising type-1 diabetic mice in experimental stroke but there are studies that employed type-1 diabetic rats (Ning et al., 2012; Toung et al., 2000; Yan et al., 2012). However, these studies involving type-1 diabetic rats do not report survival and so it is not possible to judge mortality rates. Studies that investigated type-2 diabetes and stroke utilise the C57BLKS *db/db* mouse strain, which has a mutation on the leptin receptor. This strain also develops obesity and hyperphagia (Chen et al., 1996), which is not associated with the NOD/ShiLtJ strain used in this current investigation. Tureyen *et al* found mortality to be 75% at 2 hrs of occlusion and 31% at 45 minutes of occlusion in female C57BLKS mice (Tureyen et al., 2011), while Vannucci *et al* found mortality to be at 75% in male and 40% in female C57BLKS mice at 30 minutes of occlusion (Vannucci et al., 2001).

Although these studies show diabetic strains can have high cases of mortality, none have mentioned increased thirst as a contributing factor to mortality or measured water intake.

BPH/2 mice are a potential hypertensive model and progesterone was neuroprotective when compared to non-treated hypertensive animals in our small and limited investigation. Overall, progesterone was found to be beneficial and this is consistent with the findings of Kumon *et al*, who found progesterone to be neuroprotective in spontaneous hypertensive rats (Kumon et al., 2000). Spontaneously hypertensive rats and the related stroke-prone hypertensive rats are the most widely used hypertensive animals in stroke research, a choice that has been criticised due to the animals' limited representation of processes involved in human hypertension (Pinto et al., 1998). BPH/2 mice represent another possible model of hypertension, one that is rennin-independent, while spontaneous hypertensive rats are rennin-dependent and so BPH/2 provides a model of a different type of human hypertension.

We hypothesised co-morbidity can affect the efficacy of progesterone treatment in outcomes. Our investigation using different co-morbid models supports this, as there was no apparent benefit found in aged animals, but hypertensive animals did benefit from progesterone treatment. However, care must be taken when interpreting results from these co-morbid animals due to high mortality in aged animals and low animal numbers of hypertensive animals in comparison groups. The use of co-morbid models is difficult, considering the welfare and survival of animals when conducting stroke research, as shown in this investigation. However, there is a real need to investigate efficacy of neuroprotectants in these animals due to the common co-morbidities present in patients (Ankolekar et al., 2012). The presence of co-morbidities, such as hypertension, might attenuate

neuroprotective effects, as seen with the putative neuroprotectant NXY-059 (Bath et al., 2009). There has only been one study conducted previously involving hypertensive rats (Kumon et al., 2000), and none in diabetic animals involving progesterone and stroke prior to this investigation and so more studies involving such animals are needed.

Meta-analysis of individual animal data found that, whilst progesterone might reduce ischaemic lesion volume, it also appears to increase the incidence of stroke-related death, particularly in young ovariectomised females. Progesterone's negative effects in this sub-group, highlights the fact that endogenous hormone background needs to be taken into account in experimental stroke studies. This is partly supported by the findings in the *in vitro* OGD model of ischaemia, conducted in this investigation, in which progesterone reduced viability and increased cell death, in both microglial and neuronal cells compared to non-treated cells.

The finding that progesterone appears to reduce lesion size but increases death is important since most pre-clinical studies only report the former. This may not be surprising since such studies are usually small and, therefore, do not have sufficient statistical power to individually assess death. However, the issue of low numbers of deaths also applies to clinical trials, and they typically report both death alone, and the combination of death and poor non-fatal outcome (lesion size in this study). The problem is that published preclinical studies do not routinely report death rates and that the effect of progesterone on survival has only now been revealed through analysis of IAD by contacting authors. Several potential neuroprotectants have been reported to be protective in pre-clinical studies but later proved to be hazardous in clinical trials; these include DCLHb, enlimomab, selfotel and tirilazad (2000; Davis et al., 2000; Furuya et al., 2001; Saxena et al., 1999). It is interesting to speculate on whether these

agents increased death in pre-clinical studies, and whether clinical trials would have proceeded if IAD meta-analyses had been performed beforehand. Pre-clinical studies and analysis should strive to reflect the design of clinical trials to screen out ineffective and even deleterious interventions before reaching the stage of human testing. Nevertheless, IAD meta-analyses of pre-clinical studies may not successfully predict the results of clinical trials. In the only other IAD meta-analysis performed to date, NXY-059 was found to reduce stroke lesion volume but it failed to be effective in clinical trial (Bath et al., 2009; Diener et al., 2008; Shuaib et al., 2007a). It is essential that pre-clinical studies are analysed rigorously, including performing IAD meta-analysis, before clinical trials are initiated. Also, the size and design of pre-clinical studies need to better reflect the human situation, including the use of compromised animals.

The majority of data in the meta-analysis involved young male animals and few related to aged males, or females. Further experiments in older animals are required before the translational potential of progesterone treatment can be predicted. Nevertheless, the data suggest that progesterone may be hazardous to young ovariectomised females. Although, as mentioned, there are very few studies involving female animals and the majority of these have been treated prior to stroke onset and only post-stroke treatment is relevant to human stroke, so that many of the experiments do not really contribute data relevant to the decision on whether it is appropriate to take this treatment into patients. More experimental studies involving female animals, which have been dosed after the initiation of stroke, preferably over different clinically relevant time windows, are needed.

The findings of this meta-analysis suggest that clinical trials, for any potential neuroprotectant, should not be commenced until an IAD-based meta-analysis of pre-clinical data has been performed. Meta-analysis should include death, and combined death and lesion volume as outcomes, since interventions may have both positive and negative effects. To enable such analysis, authors of pre-clinical studies should be encouraged to share their data with IAD pooling projects as is common practice in clinical medicine.

A possible alternative to progesterone as a potential neuroprotectant for clinical trials is its metabolite allopregnanolone. The metabolite has been found to have greater neuroprotective properties than progesterone (Sayeed et al., 2006; Sayeed et al., 2009). It has advantages of not being associated with female reproduction and so male patients may be more accepting of it in clinical trials. Although, there have only been a handful of studies involving allopregnanolone and neuroprotection. This metabolite still requires further investigation in order to determine its efficacy and safety as a neuroprotectant.

This investigation has clarified some issues and expanded our understanding on the neuroprotective properties of progesterone. However, these findings indicate further investigation is still required before progesterone can be considered for use in clinical trials as a neuroprotectant in stroke. The neuroprotective mechanisms of progesterone are still not fully understood and needs further exploration. In the meta-analysis of IAD, progesterone reduced lesion size but there was an increase of death in young ovariectomised female animals. However, there is a lack of studies involving female animals, particularly ones that treat animals after the onset of stroke. Therefore, more studies involving young female animals are needed to confirm, whether or not,

progesterone increases mortality in this subpopulation. Also, the meta-analysis reveals a need to utilise more co-morbid animals in studies, as currently they under-represent the demographic of stroke patients with co-morbidities. Pre-clinical studies need to be designed, so they are more clinically relevant, including consideration in terms of most effective dose, delivery method and window of opportunity available in the clinical setting.

7. APPENDIX

7.1 Pharmacokinetics Method development

The progesterone enzyme-linked immunosorbant assay (ELISA) kit from Alphadiagnostic was first evaluated by measuring plasma with and without spike concentrations of progesterone. False positives were found and sample purification methods, such as serum albumin/IgG removal and ether extraction did not produce accurate results. Samples at this stage only received one round of ether extraction due to the inability to measure progesterone accurately to determine ether extraction efficiency. It was not until adopting the progesterone enzyme immunoassay kit from Cayman Chemicals, after it was determined to be accurate in plasma samples before the ether extraction protocol for brain homogenate could be refined.

7.1.1 Progesterone ELISA kit from Alphadiagnostic

The ELISA kit from Alphadiagnostic (San Antonio, USA) was first evaluated to quantify progesterone levels in mouse serum. The principle of the assay involves the competition of progesterone in a sample or standard against horseradish peroxidase enzyme linked progesterone (progesterone-HRP

conjugate) for a limited number of binding sites on antibodies (anti-mouse IgG) bound on coated plates. In this solid phase system the antibody-bound progesterone will be retained, while unbound progesterone will be removed after washing. The added tetramethyl benzidine substrate is mixed with the antibody bound progesterone-HRP conjugate, which results in the development of a colour reaction (Blue in this case). After a short incubation (30 minutes with this assay) the enzyme reaction is stopped, by adding a stopping solution (blue turning to yellow) and the intensity of the colour (yellow) is measured with a plate reader. The intensity of colour is inversely proportional to the concentration of progesterone in the sample or control, and was read using a plate reader at 450 nm (Dynex Technologies MRX II).

However, it became evident that the kit was showing false positives in plasma controls without added progesterone. The method was reviewed, and no source of procedural error was found. Manufacture guide-lines stated that the assay has not been tested using animals, however it led on to state because progesterone is identical in all species, it can be adapted for animal use and that cross-linking with different protein complexes is unlikely. Yang Q et al 2006 (Yang et al., 2006) successfully used the progesterone assay in mouse serum without mentioning any additional purification steps. Due to irregularities in plasma controls (table 7.1.1), it became evident that a purification step would be required in order to remove any interference and so ether extraction was used. At this point in time, ether extraction was still in development and samples only undergone one round of extraction (see ether extraction development). There was a reduction in anomalous readings in ether-extracted plasma so that a reading was possible (table 7.1.2). However, a false positive was still present in plasma. It was possible that anti-mouse IgG were not only

binding to the enzyme-linked progesterone in the kit but also to mouse proteins thereby resulting in false positive readings. Ether extraction of plasma samples did not significantly remove all interference. The protocol was checked to see if the source of interference was due to contamination from equipment, reagents or poor experimental protocol. However, false positives were still present in controls. The addition of 1M hydrochloric acid (HCL) was added to the samples prior to the addition of ether in the ether extraction protocol with the intention that any proteins would be denatured to prevent IgG antibody from binding them (final concentration of HCL, after being added to the sample was not determined). This step did not prove to be effective and results were still similar (table 7.1.3).

Table 7.1.1: Plasma controls with Alphadiagnostic assay

Progesterone (ng/ml)	Reading
Control	38.83
4	38.06
40	35.16

Plasma controls and spiked plasma with different concentrations of progesterone measured with Alphadiagnostic progesterone assay. Readings are means of two independent assays.

Table 7.1.2: Plasma controls through ether extraction with Alphadiagnostic assay

Progesterone (ng/ml)	Reading
Control	16.59
4	20.26
40	64.63

Plasma controls and spiked plasma with different concentrations of progesterone measured with Alphadiagnostic progesterone assay after ether extraction. Readings are means of two independent assays.

Table 7.1.3: Plasma with HCL and ether extraction

Progesterone (ng/ml)	Reading
Control	18..69
4	29.57
40	54.43

Plasma controls and spiked plasma with different concentrations of progesterone measured with Alphadiagnostic progesterone assay after HCL and ether extraction. Readings are means of two independent assays.

7.1.2 Serum albumin/IgG removal

Body fluids have a high abundance of serum albumin and immunoglobulins. Serum albumin and immunoglobulins (IgG being the most abundant) can constitute approximately 60-97% of the total serum protein (Ahmed N, 2003). As this is a high abundance of proteins, it is probably the main contributing factor to our false positive results. The

addition of HCL may not have helped as serum albumin is a constituent in serum, that helps in buffering pH and, therefore, it is possible that it could neutralise the HCL used but could not be verified at the time. Also, HCL may denature proteins into fragments thereby making them more susceptible to binding by antibodies. A ProteoExtract albumin/IgG removal kit from Calbiochem (Darmstadt, Germany) was used to evaluate the removal of plasma/serum albumin and IgG. The kit consists of both albumin and IgG removal columns, which can be used either independently, or connected in a row with the IgG column downstream from the Albumin removal column. It is claimed that more than 80% of both albumin and IgG can be removed using the kit. The albumin removal column, despite being optimised for human serum, can be used for mouse plasma/serum and removes 72% of albumin in mouse samples compared to 86% in human serum. IgG removal column contains protein A, a highly stable protein receptor produced by *Staphylococcus aureus*. Protein A binds to the Fc receptor region of IgGs from a large number of species.

Ether extraction of control samples, followed by albumin and IgG removal did remove more false positive values as compared to ether extraction alone but were slightly more than expected (table 7.1.4). The time and number of steps required from ether extraction to albumin/IgG removal was also too impractical for a large number of samples and so another alternative was sought to measure progesterone.

Table 7.1.4: Plasma albumin/IgG removal and ether extraction

Progesterone (ng/ml)	Reading
Control	6.05
4	8.52
40	54.63

Plasma controls and spiked plasma with different concentrations of progesterone measured with Alphadiagnostic progesterone assay after Albumin/IgG removal and ether extraction. Readings are means of two independent assays.

7.1.3 Progesterone enzyme immunoassay kit from Cayman Chemicals

The progesterone enzyme immunoassay (EIA) kit from Cayman chemicals works in a similar way to the one from Alpha diagnostic and uses mouse monoclonal antibodies bound on coated plates. However, these bind to progesterone-specific rabbit antiserum, therefore eliminating the problems with anti-mouse antibodies binding to mouse proteins such as immunoglobulins. The assay is based on competition between progesterone and an acetylcholinesterase (AChE) conjugate (progesterone tracer) for a limited number of progesterone-specific rabbit antiserum binding sites. Due to the concentration of the kit progesterone tracer being held constant and the progesterone concentration in sample varying, the amount of progesterone tracer that is able to bind to the rabbit antiserum is inversely proportional to the concentration of progesterone in the well. Rabbit antiserum-progesterone (free or tracer) complex binds to the mouse monoclonal anti-rabbit IgG attached to the well. The plate is washed, to remove unbound reagents and the addition of Ellman's Reagent

(contains substrate to AChE) results in an enzymatic reaction with the production of a strong yellow colour which absorbs strongly at 412nm (405nm was the nearest available wavelength to 412nm for reading the plate, but the assay can be read from 405-420nm). Intensity of colour is inversely proportional to progesterone present. This can be represented by calculating % sample or standard bound/Maximum bound ($\%B/B_0$), as recommended for the kit.

The progesterone EIA kit was evaluated at first with control plasma followed by plasma spiked with different concentrations of progesterone (table 7.1.5). The concentration readings for plasma were consistent and accurate with regard to concentration standards used to spike the samples. Brain control and brain progesterone-spiked samples were evaluated after initial ether extraction optimisation (see ether extraction development and ether extraction protocol) and were found also to be consistent (table 7.1.5).

Table 7.1.5: Progesterone-spiked plasma and brain readings with progesterone EIA kit

Progesterone (ng)	Plasma (ng/ml)	Brain (ng/g)
500	500.21 ± 5.1	501.92 ± 7.3
100	100.13 ± 0.91	101.53 ± 4.91
50	51.03 ± 2.13	50.38 ± 2.49
25	25.89 ± 2.11	26.41 ± 0.95
10	10.43 ± 0.15	11.09 ± 0.73
Control	0.89 ± 0.41	0.72 ± 0.56

Plasma/brain controls and spiked samples with different concentrations of progesterone measured with progesterone EIA. Brain samples were ether extracted and re-suspended prior to assay. Means are expressed as means ± SEM, n= 3 each concentration

7.1.4 Ether extraction development

Ether extraction of progesterone from brain homogenate was based on a protocol described by Frye *et al* (Frye et al., 1998). Progesterone was extracted by mixing ice-cold diethyl ether with brain homogenate. The water/debris layer was then flash frozen in acetone and dry ice bath, followed by the ether layer being decanted off. Ether extract was allowed to evaporate to dryness and reconstituted prior to progesterone assay. A single round of extraction (1x) yielded only 60.35% ± 22.8 extraction efficiency across 2 different concentrations from spike homogenate (table 7.1.6 for different concentration efficiencies). Therefore, further ether extraction repeats were required.

Table 7.1.6: Ether extraction 1x of progesterone from brain homogenate (%)

50 ng/ml Progesterone	25 ng/ml Progesterone
76.82	43.88

Progesterone-spiked brain homogenate (20%) ether extraction efficiency (%). Average extraction efficiency overall was $60.35\% \pm 22.8$. Background brain progesterone has been accounted for, by measuring progesterone concentration in the same brain homogenate sample separately and subtracting this from brain homogenate plus progesterone spike. Concentrations are means of two independent assays.

Ether extraction of brain homogenate by 3 consecutive extractions (3x) resulted in higher efficiency of $98.63\% \pm 1.86$ across 2 different concentrations (see table 7.1.7 for different concentration efficiencies). This was compared to the extraction efficiency for a range of standards under the same protocol without homogenate (table 7.1.8 for efficiency extraction of standards). Extraction efficiency of these standards over all was $98.94\% \pm 0.81$, a similar level of extraction. All progesterone assays for optimisation of extraction protocols was performed with a commercial kit, which proved to have worked after initial testing (Progesterone Enzyme Immunoassay Kit; Cayman Chemicals, Michigan, USA). These initial optimisation experiments were used to develop the ether extraction protocol for pharmacokinetics of progesterone in treated animals.

Table 7.1.7: Ether extraction 3x of progesterone from brain Homogenate (%)

100 ng/ml Progesterone	10 ng/ml Progesterone
97.32	99.95

Progesterone-spiked brain homogenate (20%) ether extraction efficiency (%). Background brain progesterone has been accounted for, by measuring progesterone concentration in the same brain homogenate sample separately and subtracting this from brain homogenate plus progesterone spike. Average extraction efficiency overall was $98.63\% \pm 1.86$. Concentrations are means are of two independent assays.

Table 7.1.8: Ether extraction 3x of progesterone standards (%)

Progesterone (ng/ml)	% Extraction
500	99.45
100	98.67
50	97.90
25	100.00
10	98.70

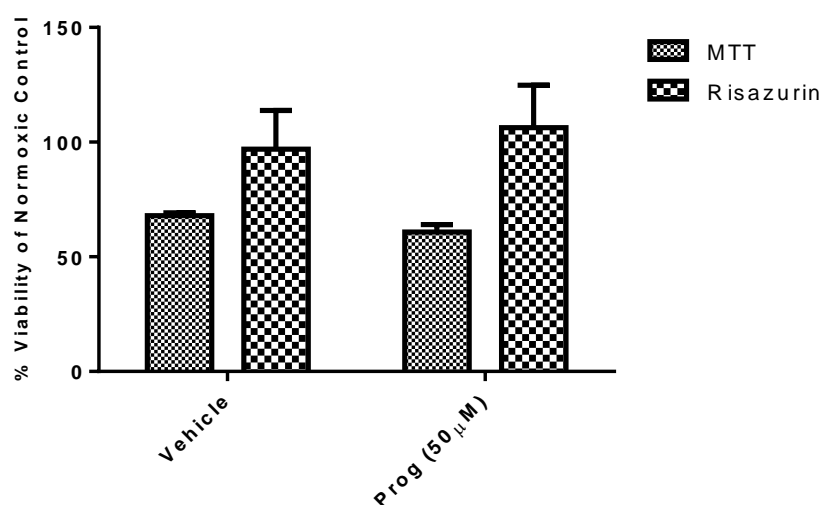
Extraction efficiency (%) of progesterone ether extracted standards. Background brain progesterone has been accounted for, by measuring progesterone concentration in the same brain homogenate sample separately and subtracting this from brain homogenate plus progesterone spike. Average extraction efficiency overall was $98.94\% \pm 0.81$ (Mean \pm SEM).

7.2. Method development for SHSY5Y viability

7.2.1 MTT viability assay

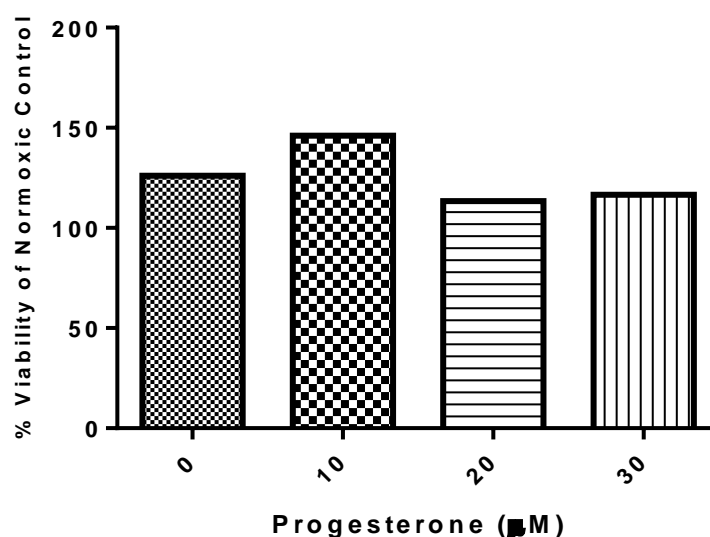
The MTT assay was found to indicate lower cell viability compared to the resazurin assay in OGD controls and progesterone-treated cells (figure 7.2.1). However, in other experiments using MTT with increasing progesterone concentrations in OGD conditions, the assay appeared not to be detecting cell death. This inconsistency can be attributed to the step in the method when aspirating cell culture media after incubation with MTT. The cytotoxicity of MTT formazan makes it difficult to remove cell culture media from culture wells. MTT formazan needles form causing cells to float, allowing formazan to be aspirated with culture media, resulting in inconsistent results (higher ischaemic controls to normal, figure 7.2.2).

Figure 7.2.1: Comparison of MTT and resazurin assay controls after 2 hrs of OGD for SHSY5Y cell line



Cell viability is expressed as % normoxic control. Cultures consisted of vehicle and progesterone (prog, 50 µM) treated cells and incubated for 2hrs in OGD conditions. Cell viability was then measured with Neutral Red assay. Data are expressed as means \pm SEM (n=3). Analysis with two-way ANOVA showed a significant trend for the MTT assay to detect lower levels of viability compared to the resazurin one [$F(1,6)=31.00$, $P=0.0014$].

Figure 7.2.2: SHSY5Y viability after 3 hrs of OGD using MTT assay

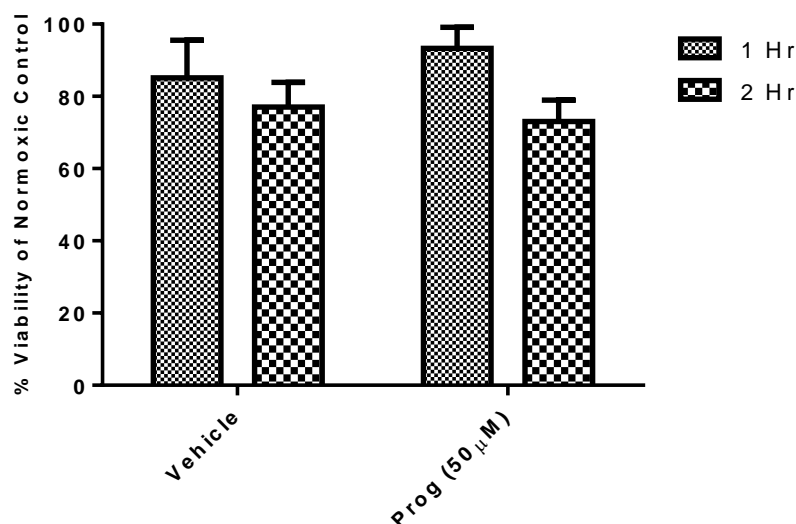


Cell viability is expressed as % of normoxic control. Cultures were incubated increasing concentrations of progesterone (0-30µM) over 3hrs of OGD. Cell viability was measured with MTT assay. All treatment wells contained 0.1% DMSO. Data are expressed as means of eight repeats on one occasion. Analysis with one-way ANOVA found no significant effect of progesterone [$F(1,28)= 0.91$, $P= 0.4504$].

7.2.2 Resazurin viability assay

The resazurin assay requires an incubation period for the dye to be converted to resorufin. Incubation times of 1 and 2 hrs were evaluated to determine the optimum incubation time under OGD conditions (figure 7.2.3). There was no significant difference found between incubation times. One hour incubation was, therefore, used in further resazurin experiments due to the reduced time required to complete the assay.

Figure 7.2.3: SHSY5Y viability after 2hrs of OGD, followed by incubation with resazurin at 1 and 2hrs before assay

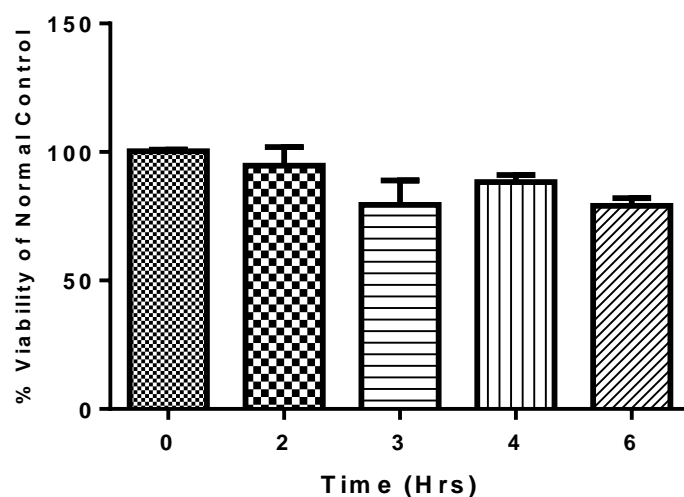


Cell viability is expressed as % normoxic control. Cultures consisted of vehicle and progesterone (prog, 50 µM) treated cells and incubated for 2 hrs in OGD conditions. Cell viability was then measured with resazurin assay after 1 hr and 2 hr incubation with resazurin. Data are expressed as means \pm SEM (n=3). Analysis with two-way ANOVA found no significant differences between 1 and 2 hr incubation with resazurin [$F(1,12)= 3.57$, $P= 0.0833$].

7.2.3 Viability of SHSY5Y using the resazurin assay

SHSY5Y cells were exposed to increasing OGD conditions to reduce viability. However, no significant reduction of viability was found over 6 hrs (figure 7.2.4).

Figure 7.2.4: SHSY5Y viability of OGD controls with increasing incubation time using resazurin assay

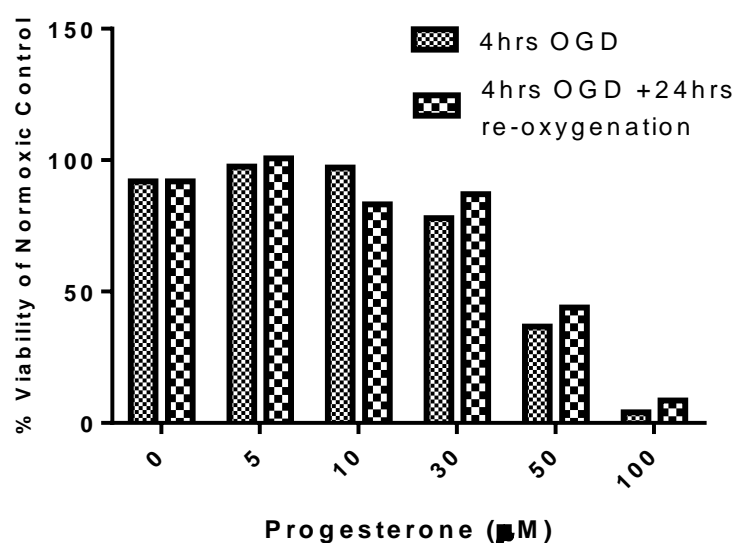


Cell viability is expressed as % of normoxic control. Cultures were subjected to increasing periods of OGD. Cell viability was then measured after 1hr incubation with resazurin. All treatment wells contained 0.1% DMSO. Data are expressed as means \pm SEM (times 3 and 6 hrs $n=4$, 2 and 4 hrs $n=3$). One-way ANOVA revealed no significant differences from control [$F(4,12)=0.11$, $P=0.1142$].

Due to the lack of cell death caused by OGD conditions, 24 hrs of re-oxygenation after OGD was introduced to mimic reperfusion injury. Cells were exposed to OGD with progesterone for 4 hrs, with and without re-oxygenation. Because no significant differences were found between OGD time points between 2-6 hrs, 4 hrs of OGD was chosen followed by re-oxygenation as a compromise to complete the experiment in the time available and to observe any effects from progesterone. It was not known at the time if re-oxygenation would increase cell death. Therefore, as a precaution, progesterone concentrations were included, in case a reduction

of viability was achieved. However, the addition of re-oxygenation following OGD did not increase cell death compared to OGD alone, but unexpectedly, increasing progesterone concentrations did reduce cell viability (figure 7.2.5). Also, viability of non-treated controls in figure 3.5 (91.89 %) are greater than figure 3.3 (85.09 %) for the same time in OGD conditions and incubation with resazurin. A positive control for cell killing (e.g. hydrogen peroxide or staurosporine) may have been useful as a comparison to OGD, but was not available at the time.

Figure 7.2.5: Viability of SHSY5Y cells exposed to increasing concentrations of progesterone during 4 hrs of OGD, with or without 24 hrs of re-oxygenation using resazurin assay

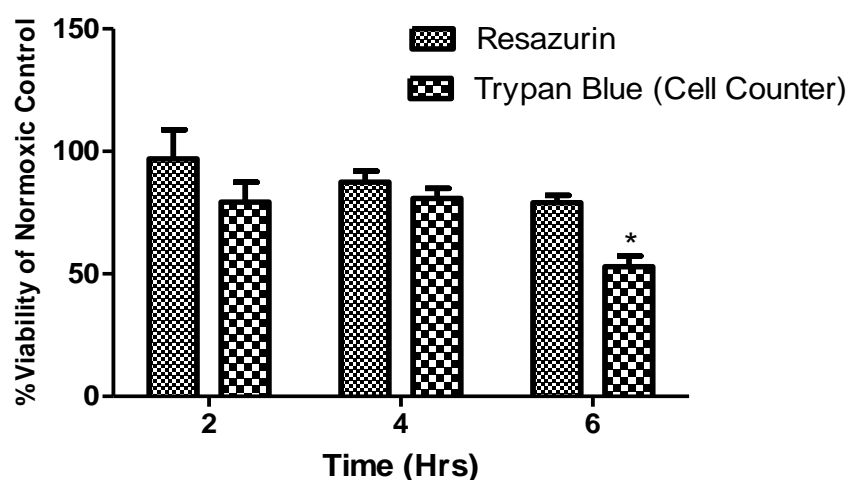


Cell viability is expressed as % of normoxic control. Cultures were either exposed to 4 hrs of OGD alone or followed by 24 hrs of re-oxygenation in SFM. All treatment wells contained 0.1% DMSO. Cell viability was then measured with resazurin assay after 1hr incubation with resazurin. Data are expressed as means of 8 repeats in one experiment.

7.2.4 Viability comparison of resazurin assay with trypan blue (cell counter) in SHSY5Y

The results of the resazurin assay were then compared to those generated in the trypan blue assay with an automated cell counter. The assays were compared with OGD controls over increasing periods of OGD (figure 7.2.6). The trypan blue with automated cell counter assay at 6hrs was found to indicate a lower viability compared to the resazurin assay. Due to the resazurin assay showing no significant drop in viability of SHSY5Y cells due to OGD, the trypan blue with cell counter assay was used to measure viability in subsequent experiments. Trypan blue selectively colours dead cells blue by its negatively charged chromophore, which does not interact with the cell unless the membrane is damaged and the membrane potential collapsed. In apoptosis, the integrity of the cell membrane is maintained until late in the process (Bohm and Schild, 2003), and so a clear indicator of cell death when it reaches this threshold. Other assays based on the biochemical or enzyme activity of mitochondria, such as MTT or resazurin is dependent on metabolic activity. But if a cell is metabolically quiet it may not necessarily mean it is not viable.

Figure 7.2.6: SHSY5Y viability of OGD controls over time: comparison of resazurin and trypan blue assays



Cell viability is expressed as % of normoxic control. Cultures were either exposed to OGD conditions over time. Cell viability was then measured with either resazurin or trypan blue assay. Data are expressed as means \pm SEM (Resazurin $n=2-4$, Trypan Blue $n=3-6$). Two-way ANOVA shows there was a significant for trypan blue assay to detect lower viability compared to resazurin [$F(1,14)=11.55$, $P=0.0043$]. Bonferroni post-hoc analysis revealed that the trypan blue assay showed significantly reduced viability at 6hrs of ischaemia ($P<0.01$).

7.3.1 Invitation letter template to authors for individual animal meta-analysis collaboration

Dear

I am writing to you on behalf of my group in the hope that you will be willing to share your raw animal data from your work on progesterone in experimental stroke.

We are performing an individual animal data meta-analysis of all the available data on the use of progesterone as a neuroprotectant in stroke. At present there has only been two clinical trials aimed at using progesterone as a neuroprotectant, this suggesting an improved outcome and good safety tolerance after traumatic brain injury. There have been no clinical trials however that have examined the effect of acute administration of progesterone after stroke so far. In order to design future clinical trials aimed at assessing the neuroprotective potential of progesterone, further experimental investigation is needed. In order to assess how we can improve and identify the key factors that require further investigation we would like to look at the individual animal data. Individual subject data meta-analyses are the gold standard and offer significant advantages over using summary data, not least since subgroup analyses may be performed.

I should add that my group has significant experience in performing systemic reviews, both clinical and pre-clinical, and using individual subject and summary data. Recently, we have performed one of the first (if not first) meta-analyses using individual animal data of NXY-059 (Bath et al., 2009). In particular, we understand the need for maintaining confidentiality when working with other people's data. Also we do not identify individual data from authors. Rather, we put together all the data

from the different studies and then try to explore why it gives the results it does and also whether differences in subgroups are important.

We believe that the results may help clarify the similarities and differences in existing progesterone animal studies. I do hope that you will be able to support this potential project through sharing the raw data from your studies of progesterone in stroke.

If you are willing, I will send you the kind of data we are looking for but broadly this will relate to characteristics (age, weight, sex), drug administration (dose, timing) and the effect (lesion size, behavioural effects) animal by animal.

At present we have received much interest in our investigation and have already received individual animal data from ten published studies, as well as unpublished data. We are hopeful by the response so far and even now we are expecting to receive even more data. On behalf of our group we would like to encourage you to be a part of this endeavour and would be grateful with whatever data from your progesterone animal studies you can provide. We hope that inevitably we will be able to even further clarify our understanding of progesterone in stroke.

Many thanks for considering this request.

Raymond Wong, MSc

David Ray, PhD

Claire Gibson, PhD

Philip Bath, FRCP

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Web: www.nottingham.ac.uk/stroke-medicine/

ENOS trial: www.enos.ac.uk/

7.3.2 Document outlining individual animal data meta-analysis to authors

Progesterone in experimental stroke: assessment of safety and efficacy using data from individual animals in completed preclinical studies (PISA)

Raymond Wong,¹ MSc; Michael Tracy,¹ MSc; David Ray,¹ PhD; Claire Gibson,² PhD; Philip Bath,¹ FRCP

Institute of Neuroscience,¹ University of Nottingham, and School of Psychology,² University of Leicester

Introduction

At present there has only been two clinical trials aimed at using progesterone as a neuroprotectant, suggesting improved outcome and good safety tolerance after traumatic brain injury (Wright et al., 2007; Xiao et al., 2008). However, there have been no clinical trials examining the effect of acute administration of progesterone after ischaemic stroke. Several studies assessing the safety and efficacy of progesterone in preclinical models of stroke have been performed, (Alkayed et al., 2000; Chen and Chopp, 1999; Chheng-Orn Evans et al., 2004; Gibson et al., 2005b; Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002; Murphy et al., 2000; Roof et al., 1996) and these have been integrated in a systematic review based on published summary ('group') data (Gibson et al., 2008a). However, this systematic review suggests that key important questions remain to be answered, including efficacy by sex and age, and dose and time responses to progesterone. In order to assess how to improve and identify the key factors that require further investigation, a meta-analysis using individual animal data (IAD) is

required. Individual subject data meta-analyses are the gold standard and offer significant advantages over using summary data, not least since subgroup analyses may be performed. The results from the IAD meta-analysis will be used to inform the design of future preclinical studies of progesterone in stroke.

Methods

Study Identification

Experimental studies examining the effect of progesterone or progesterone metabolite on lesion size and functional recovery in animal models of stroke will be identified from electronic searches (e.g PubMed), references lists in known published studies, (Alkayed et al., 2000; Chen and Chopp, 1999; Chheng-Orn Evans et al., 2004; Gibson et al., 2005b; Gibson and Murphy, 2004; Jiang et al., 1996; Kumon et al., 2000; Murphy et al., 2002; Murphy et al., 2000; Roof et al., 1996) and a systematic review of them (Gibson et al., 2008a), reference lists in published review articles and personal communication with authors of all of these.

Study Selection

Studies must be controlled (whether randomised or not) and involve exogenous administration of progesterone or progesterone metabolite in experimental stroke. Stroke models may include transient or permanent focal cerebral ischemia. Studies, whether published or unpublished, will be included if they contain data on lesion volume, vital status, and/or functional outcome.

Initiation of the Collaboration

The Chief Investigator of each identified study will be contacted as to whether they are willing to join the collaboration and share data on individual animals.

Data extraction

The following information will be sought from each Chief Investigator: animal species, experimental model of brain injury (permanent or transient), design, randomisation/pseudo-randomisation, blinding of surgeons to treatment, whether the outcomes were accessed blinded to treatment.

Study Characteristics

The individual animal data will include (accepting that not all variables will have been recorded for each study):

- Sex/hormonal state
- Species
- Number of Animals
- Age
- Weight
- Model (global/focal, permanent/transient)
- Lesion/infarct volume (total, cortical and sub-cortical if available)
- Neuroscore (Bederson and type)
- Motor impairment and scale

- Functional outcome and scale (foot fault, Rotarod, Morris water maze, etc)
- Temperature during treatment (different time points, max and min)
- Vital Status (Death)
- MRI (lesion values)
- Deaths (spontaneous or early culling for poor health)
- Physiological variables (temperatures, blood pressure)

Information on treatment will also be obtained including:

- Time to treatment in relation to onset of ischemia
- Treatment- Number of Doses, interval or duration if continuous
- Progesterone load mg dose
- Progesterone maintenance dose
- Progesterone concentration in blood
- Time at which concentration measured
- Form of occlusion
- Occlusion time
- Plasma concentration
- Type of Anaesthetic
- Reperfusion start relative to onset time

Methodological Quality

Methodological quality of each study will be assessed using a 9 point score based on the STAIR (1999) rating (STAIR rating is a 8 point score, however with addition of masked surgery it is now 9 points) (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). A point is given for the written evidence for each of the following criteria:

- Presence of randomisation (0.5 given for pseudo-randomisation),
- Monitoring of physiological parameters,
- Assessment of dose-response relationship,
- Optimal time window assessment,
- Masked outcome measurement,
- Outcome assessment of days 1-3 and 1-30,
- Combined measurement of lesion/infarct volume,
- Functional outcome
- Masked surgery (addition to STAIR rating)

Data Analysis

IAD and summary data will be analysed together followed by IAD analysed separately. Analysis of both summary and IAD will involve random effect models to produce standardised mean differences (SMD, for continuous or ordinal data, www.cochrane-net.org/openlearning/html/modA1-4.htm, 25/11/08) and 95% confidence intervals (95% CI). Random effects models will be used since biological heterogeneity is expected due to the expected varied nature of studies involving different species, model of ischaemia, time to treatment and doses of progesterone. The statistical heterogeneity will be calculated using I^2 statistic and presence of publication bias will be assessed by funnel plot and Egger's test (Egger et al., 1997).

In the case of IAD analysis, the data will merged into an Excel spreadsheet with each row containing data for an individual animal. Multilevel models will be built to compare progesterone with control taking into account differences between trials. The infarct volume will be standardised (score-mean/standard deviation) to account for any differences in between brain and infarct sizes in different species. Motor impairment will be similarly

standardised since difference scales is expected (although most be derived from Bederson *et al*) (Bederson *et al.*, 1986). The coefficients for dose given will be per 100mg kg⁻¹. Analyses of dose/concentration and concentration/response relationships for progesterone (if measured) will be identified reflecting steady state levels, typically at 24 hours.

Experience

The team are experienced at performing preclinical and clinical systematic reviews, including with summary data and individual subject data. Completed reviews include:

- Preclinical – summary data: progesterone, oestrogen, nitric oxide, nitric oxide synthase inhibitors (Gibson *et al.*, 2008a; Gibson *et al.*, 2005c; Willmot *et al.*, 2005a; Willmot *et al.*, 2005b)
- Preclinical – individual animal data: NXY-059 (Bath *et al.*, 2008)
- Clinical - summary data: see Cochrane Library, heparin (Bath *et al.*, 2002; Bath *et al.*, 2000)
- Clinical - individual patient data: dipyridamole, occupational therapy (Leonardi-Bee *et al.*, 2005; Walker *et al.*, 2004)

Publication Strategy

The findings will be published under the group name of 'Progesterone In Stroke Animals' (PISA).

The Collaboration

The collaboration will comprise:

Project secretariat:

Philip Bath: Chief Investigator (and PhD supervisor)

Raymond Wong: Administer project (and PhD student)

Michael Tracy: Bio-medical statistician (and PhD student)

David Ray: Scientific advisor (and PhD supervisor)

Claire Gibson: Scientific advisor

Collaborators:

All the Chief Investigators

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